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Reductive Dechlorination of Polychlorinated Biphenyls in Marine Sediments

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The goal of this proposal was to provide a basic understanding of the dehalogenating processes extant in coastal sediments using PCBs as a model system. An understanding of the anaerobic microbial physiology involved in such a process will ultimately provide information on the factors that enhance and limit the process, and enable us to determine which portion of the degradative process would be potentially amenable to biotechnological enhancement. Milestones of this research include: i)discovery of meta and ortho dechlorination of Aroclor 1260, ii) development of the first defined microbial population that reproducibly ortho-dechlorinates PCB congeners, iii) development of additional cultures which specifically para- or meta-dechlorinate PCBs; iv)development of the first anaerobic cultures that reductively *ortho*-and *para*-dechlorinate PCBs in a completely defined medium in the absence of sediment; and v) 16s rDNA-based phylogenetic characterization of individual species in *ortho*-, *para*-, and *meta*-PCB-dechlorinating cultures. As a result of developments in this project, we have demonstrated that by combining enrichment techniques with molecular monitoring it is now possible to develop highly defined and selective PCB-dechlorinating microbial populations in a defined minimal medium.

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OBJECTIVE: The goal of this proposal was to provide a basic understanding of the dehalogenating processes extant in coastal sediments using PCBs as a model system. An understanding of the anaerobic microbial physiology involved in such a process will ultimately provide information on the factors that enhance and limit the process. Results of this research will enable us to screen for PCB-dechlorinating potential in sediments using species-specific probes, and enable us to determine which portion of the degradative process would be potentially amenable to biotechnological enhancement. An understanding of microbial processes that catalyze PCB dechlorination will also facilitate Navy management decisions concerning both remedial site prioritization and appropriate remedial strategies.

APPROACH: In a collaborative effort between PIs at the University of Maryland Biotechnology Institute and Medical University of South Carolina, classical enrichment culture techniques were combined with 16S rDNA gene probe molecular monitoring to develop highly defined PCB dechlorinating consortia of microorganisms. By using single congeners and selective inhibitors to enrich for individual dechlorinating species, the PIs have identified a number of microbes that are associated with dechlorination. Putative dechlorinating species are currently being isolated for further physiological characterization.

ACCOMPLISHMENTS: In the course of the past three years of ONR funded research, the P.I.s have effectively identifed potential PCB-dechlorinating microbes without isolation. Milestones of this research include: i) discovery of meta and ortho dechlorination of Aroclor 1260, ii) development of the first defined microbial population that reproducibly ortho-dechlorinates PCB congeners, iii) development of additional cultures that specifically para- or meta-dechlorinate PCBs; iv) development of the first anaerobic cultures that reductively *ortho*- and *para*-dechlorinate PCBs in a completely defined medium in the absence of sediment; and v) 16s rDNA-based phylogenetic characterization of individual species in *ortho*-, *para*-, and *meta*-PCB-dechlorinating cultures.

CONCLUSIONS: As a result of developments in this project, we have demonstrated that by combining enrichment techniques with molecular monitoring it is now possible to develop highly defined and selective PCB-dechlorinating microbial populations in a defined minimal medium. This approach will enable us to isolate species that catalyze PCB dechlorination by minimizing competition with faster growing non-PCB dechlorinating microbes. Ultimately, isolation of the PCB-dechlorinating microorganisms will enable us to: i) determine physiological parameters that enhance or limit the dechlorination process; and ii) design species-specific molecular probes to screen for PCB-dechlorinating potential in cultures and *in situ*.

PATENT INFORMATION:

Bacterial Dechlorination of Polychlorinated Biphenyls (PCBs). Mary Berkaw, Leah A. Cutter, Margaret Elberson, Tracey Holoman, Harold D. May, Tracey Holoman, Kevin R. Sowers. Currently being reviewed by tech transfer departments at both universities.

PUBLICATIONS AND ABSTRACTS(for total period of grant):

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Microbial Dechlorination of 2,3,5,6-Tetrachlorobiphenyl under Anaerobic Conditions in the Absence of Soil or Sediment

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Bacterial enrichment cultures developed with Baltimore Harbor (BH) sediments were found to reductively dechlorinate 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB) when incubated in a minimal estuarine medium containing short-chain fatty acids under anaerobic conditions with and without the addition of sediment. Primary enrichment cultures formed both *meta* and *ortho* dechlorination products from 2,3,5,6-CB. The lag time preceding dechlorination decreased from 30 to less than 20 days as the cultures were sequentially transferred into estuarine medium containing dried, sterile BH sediment. In addition, only *ortho* dechlorination was observed following transfer of the cultures. Sequential transfer into medium without added sediment also resulted in the development of a strict *ortho*-dechlorinating culture following a lag of more than 100 days. Upon further transfer into the minimal medium without sediment, the lag time decreased to less than 50 days. At this stage all cultures, regardless of the presence of sediment, would produce 2,3,5-CB and 3,5-CB from 2,3,5,6-CB. The strict *ortho*-dechlorinating activity in the sediment-free cultures has remained stable for more than 1 year through several transfers. These results reveal that the classical microbial enrichment technique using a minimal medium with a single polychlorinated biphenyl (PCB) congener selected for *ortho* dechlorination of 2,3,5,6-CB. Furthermore, this is the first report of sustained anaerobic PCB dechlorination in the complete absence of soil or sediment.

Anaerobic dechlorination of polychlorinated biphenyls (PCBs) has been demonstrated in situ and with laboratory microcosms containing sediment (reviewed in reference 1a). However, sustained PCB dechlorination has never been shown to occur in the absence of soil or sediments. Morris et al. (6) demonstrated a sediment requirement for the stimulation of PCB dechlorination within freshwater sediment slurries. Wu and Wiegel have recently described PCB-dechlorinating enrichments which required soil for the successful transfer of PCB-dechlorinating activity (9). In addition, no anaerobic microorganisms that dechlorinate PCBs have been isolated or characterized, and this may be due in part to the soil or sediment requirement. The inability to isolate dechlorinating organisms or maintain dechlorination without sediment has limited biogeochemical and physiological investigations into the mechanisms of PCB dechlorination.

Dechlorination (*ortho*, *meta*, and *para*) of single PCB congeners has been observed following anaerobic incubation of Baltimore Harbor (BH) sediment under estuarine or marine conditions (2). While sediments from several sites within BH are contaminated with PCBs (1, 5), background contamination of sediment is not necessarily a prerequisite for the development of PCB dechlorination in laboratory microcosms. Wu et al. (8) recently demonstrated *meta* and *ortho* dechlorination of Aroclor 1260 when it was added to the same BH sediments. These results showed that more than one dechlorinating activity could be developed with these sediments. It has been proposed that discrete microbial populations are responsible for specific PCB dechlorinations (1a). Consistent with this idea, the *ortho* dechlorination observed with BH sediments may be catalyzed by discrete microbial populations. In addition, these organisms may be able to couple PCB dechlorination with growth. Therefore we have attempted to select for *ortho* PCB-dechlorinating organisms by enrichment under minimal conditions with high levels of 2,3,5,6-tetrachlorobiphenyl. We also speculated that given the proper conditions, a PCB-dechlorinating population could be maintained in an actively dechlorinating state in the absence of sediment. Here we report that a distinct PCB-dechlorinating activity, namely, *ortho* dechlorination, was selected for through sequential transfer initiated with sediments from BH and sustained in the absence of soil or sediment. This is the first report of sustained anaerobic PCB-dechlorinating activity in the total absence of sediment.

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MATERIALS AND METHODS

Sediment samples. Sediment samples were collected with a petite Ponar grab sampler from a subsurface depth of 9.1 m in the northwest branch of BH (39°16.8'N, 76°36.1'W). An oily slick and gas bubbles formed at the surface immediately after the sampler disturbed the sediments. Sediments had a black coloration, a gelatinous texture, and a strong petroleum odor. The combined contents of the sampler were transferred to 0.95-liter canning jars (Ball Corporation, El Paso, Tex.). The jars were filled to the top and immediately sealed with dome tops and ring seals to exclude air. The samples were stored at ambient temperature in the dark prior to use.

Culture conditions. All sterile media in these experiments included an estuarine salts medium without sulfate (E-Cl) and were prepared anaerobically in an atmosphere that contained N_2-CO_2 (4:1) as previously described by Berkaw et al. (2). Briefly, the medium contained the following constituents, in grams per liter of demineralized water: Na_2CO_3, 3.0; Na_2HPO_4, 0.6; NH_4Cl, 0.5; cysteine-HCl · H_2O, 0.25; Na_2S · $9H_2O$, 0.25; $MgCl_2$ · $6H_2O$, 0.1; $CaCl_2$ · $6H_2O$, 0.1; and resazurin, 0.001. In addition, vitamin and trace element solutions (1% [vol/vol] each) were added (7). The final pH of the medium was 6.8. Media were dispensed into anaerobic culture tubes (18 by 160 mm; Bellco Glass, Inc., Vineland, N.J.) or 150-ml serum bottles (Wheaton, Millville, N.J.) sealed with Teflon-lined butyl stoppers (The West Co., Lionville, Pa.) that were secured with aluminum crimp seals (Wheaton).

Primary sediment enrichment cultures were generated in culture tubes by adding 2 ml of BH sediment to 8 ml of sterile E-Cl medium (approximately 5% wt/vol [dry weight], sediment concentration), plus a mixture of sodium acetate, propionate, and butyrate to final concentrations of 2.5 mM each. Congener 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB) was solubilized in acetone and added to each culture to a final concentration of 173 μM (50 ppm), and this resulted in a

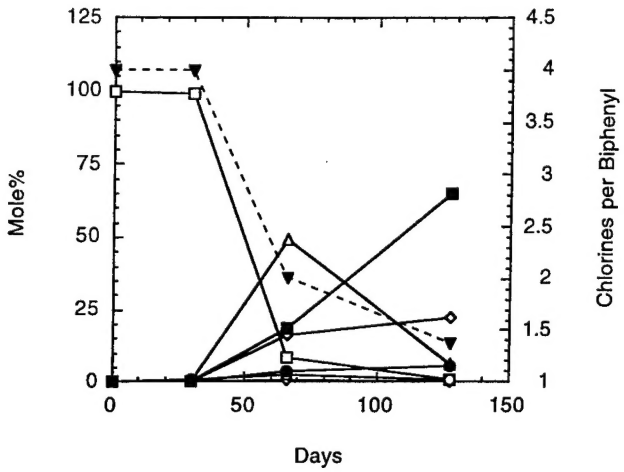

FIG. 1. Dechlorination of 2,3,5,6-CB by a primary enrichment culture with BH sediment (5.0%, wt/vol [dry weight]). Mole percent and chlorines-per-biphenyl data are from a single culture. Symbols: ■, mole percent for 3-CB; ◇, 2,5-CB; ●, 2,6-CB; △, 3,5-CB; ○, 2,3,5-CB; ◆, 2,3,6-CB; and □, 2,3,5,6-CB. ▼, chlorines per biphenyl.

0.1% (vol/vol) concentration of acetone. Cultures were incubated under strict anaerobic conditions at 30°C in the dark. Killed-cell controls were sterilized in an autoclave at 121°C for a total of 3 h (two 1.5-h treatments). Sequential transfers of sediment-containing cultures were made as follows. The entire sediment-containing culture was made into a suspension by shaking, and then the particulate matter was allowed to settle for approximately 1 min. Supernatant material was then transferred in order to minimize the amount of sediment passed to the next vessel. Sequential transfers (10% [vol/vol]) from primary enrichment cultures were made into E-Cl medium with dried BH sediment (0.1%, wt/vol [dry weight], unless stated otherwise) that was then sterilized in an autoclave at 121°C for a total of 3 h (two 1.5-h treatments). Subsequent transfers were made under identical conditions every 2 to 5 months. Sequential transfers (10% [vol/vol]) for the establishment of sediment-free cultures were made every 2 to 5 months into identical media without sediment. Following the first two transfers, the amount of sediment passed was negligible.

Spectrophotometric analysis. Growth in sediment-free cultures was monitored by measuring the increase in optical density at 600 nm (OD_{600}) with a Spectronic 20D spectrophotometer (Milton Roy, Rochester, N.Y.).

Sampling and PCB analysis. Aliquots were withdrawn anaerobically once at each time point from shaken cultures by using the reverse end of a 5-ml glass pipette (front end for sediment-free samples). Samples were extracted in ethyl acetate and passed over a Florisil-copper column as previously described by Berkaw et al. (2). Analysis was conducted with a Hewlett-Packard 5890A gas chromatograph (GC) equipped with an electron capture detector (ECD) and an RTX-1 capillary column as previously described (2). Standards for 2-, 3-, 4-, 2,3-, 2,5-, 2,6-, 3,5-, 2,3,5-, 2,3,6- and 2,3,5,6-CB were purchased from AccuStandard (New Haven, Conn.). PCB congeners were identified by retention time and quantified with a 16-point calibration curve for each congener according to the method of Berkaw et al. (2).

RESULTS AND DISCUSSION

Selection of *ortho* dechlorination. Reductive dechlorination of 2,3,5,6-CB was observed to occur in primary enrichment cultures incubated with BH sediment under anaerobic conditions. GC-ECD analysis revealed various *meta* and *ortho* dechlorination products. Large amounts of transient 3,5-CB, along with smaller amounts of 2,5-CB and 2,6-CB, were observed, while 3-CB eventually became the dominant product (Fig. 1). No dechlorination products were ever observed in killed-cell controls (sterilized sediment and media) or no-inoculum controls.

Sequential transfers (10% [vol/vol]) from the primary culture were made into E-Cl medium containing 0.1% (wt/vol [dry weight]) sterile BH sediment. Selection of *ortho* dechlorination reactions with a loss of *meta* reactions was observed after the first transfer. Further transfer resulted in cultures that exclusively *ortho* dechlorinated 2,3,5,6-CB to 3,5-CB within 50 days, with 2,3,5-CB as the only intermediate detected by GC-ECD analysis (Fig. 2). In contrast to the large accumulation of 3-CB observed in primary cultures, virtually all of the 2,3,5,6-CB was transformed to 3,5-CB in the transferred cultures, with no other end products observed. This pattern of strict *ortho* dechlorination of the 2,3,5,6-CB remained the same through five sequential transfers (made once every 3 to 5 months) beyond the primary enrichment cultures. In addition, no monochlorobiphenyl arose, even after extensive incubation lasting up to a year.

Microbial dechlorination of 2,3,5,6-CB in the absence of sediment. Sequential transfers (10% [vol/vol]) from the primary cultures containing 5% (wt/vol [dry weight]) BH sediment were made into E-Cl medium with no added sediment. By the second transfer, the sediment was no longer visible to the naked eye and a low rate of dechlorination was observed. A mixed culture dominated by blunt-end rod-shaped cells and small vibrio-shaped cells developed. Each transfer culture was started at an OD_{600} between 0.01 and 0.05 and was transferred after the OD_{600} was >0.1, regardless of the degree of dechlorinating activity. Congener 2,3,5,6-CB was added to a final concentration of 173 μM, which is significantly above its aqueous solubility limit (3). However, reasonable recoveries of the PCB, 80% on average, could be made by vigorously mixing the culture with a pipette before sampling. It was assumed that the PCBs were sorbing to or partitioning into the biomass.

The objective at this point was to detect a significant amount of dechlorination product in the sediment-free culture series. Significant amounts (mole percent) of 2,3,5-CB and 3,5,-CB began to accumulate in the fourth-sequential-transfer cultures following extensive incubation (Fig. 3). After more than 200 days, when *ortho* dechlorination had been clearly established and the culture had become more turbid (OD_{600} > 0.2), these sediment-free cultures were transferred again. This fifth-sequential-transfer culture had a shortened lag time of less than 50 days (Fig. 3). No dechlorination was observed with sterile (killed-cell) or no-inoculum controls. Only *ortho* dechlorination of the 2,3,5,6-CB to 2,3,5-CB and 3,5-CB was observed at all times with all of these cultures. Since the establishment of stable dechlorination, we have been able to routinely transfer

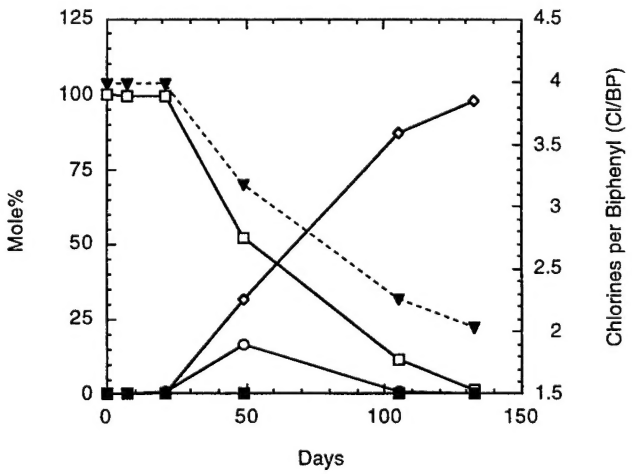

FIG. 2. Dechlorination of 2,3,5,6-CB after the first transfer of the supernatant from the primary enrichment culture into E-Cl medium with BH sediment (0.1% wt/vol [dry weight]). Mole percent and chlorines-per-biphenyl data are from a single culture. Symbols: ■, mole percent for 3-CB; ◇, 3,5-CB; ○, 2,3,5-CB; and □, 2,3,5,6-CB. ▼, chlorines per biphenyl.

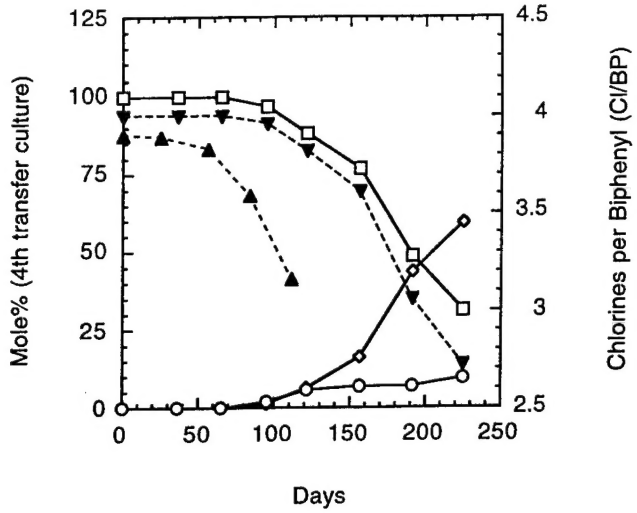

FIG. 3. Chlorines-per-biphenyl data for fourth- and fifth-sequential-transfer cultures without sediment. Mole percent data are given for the fourth-transfer culture. All data are given as the averages from duplicate cultures. Symbols: ◇, mole percent for 3,5-CB; ○, 2,3,5-CB; and □, 2,3,5,6-CB. ▼, chlorines per biphenyl of fourth-sequential-transfer culture; ▲, chlorines per biphenyl of fifth-sequential-transfer culture.

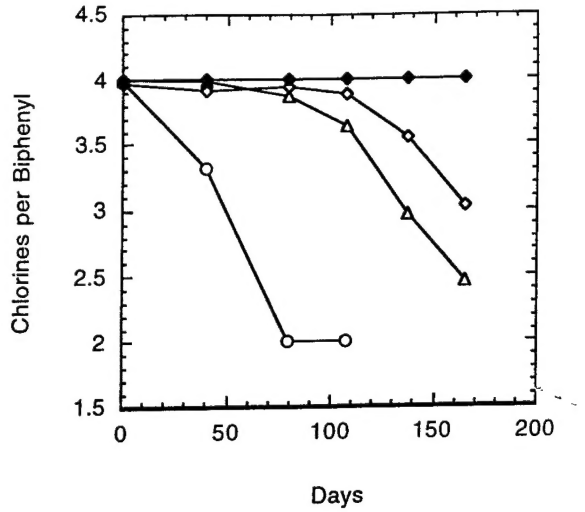

FIG. 5. Cultures with 2,3,5,6-CB and 1.0% (○), 0.1% (△), and 0.05% (□) (wt/vol [dry weight]) sterilized BH sediment. Supernatant from a 5.0% sediment culture was sequentially transferred with 1.0, 0.1, and 0.05% (wt/vol [dry weight]) BH sediment in E-Cl medium, incubated for 4 months, and transferred again under identical conditions. The data presented represent the second set of transferred cultures. The chlorines-per-biphenyl data for the killed-cell control with 1.0% sterilized BH sediment are for a single culture (◆). The data from the live BH cultures are the average of duplicates.

these cultures into identical sediment-free media and still maintain dechlorinating activity.

The appearance of dechlorination at the fourth transfer of the sediment-free cultures after an incubation period exceeding that of earlier cultures in the transfer series suggests that the transfers were made too quickly (at low cell density) during the early part of the enrichment process. OD data for a later set of active sediment-free cultures (Fig. 4) revealed that significant dechlorination does not occur until the OD_{600} exceeds 0.2. This observation supports our conclusion that the ability to maintain good dechlorination earlier on in the sediment-free enrichment series was hindered by premature transfer of the cultures at low turbidity. Perhaps the earlier transfers at lower turbidity had prevented the development of hearty dechlorinating cultures and sustainability was simply an issue of low numbers of dechlorinators among the total population. The possibility that the organisms responsible for the dechlorination needed an extensive amount of time to adjust to the altered conditions (lack of sediment) before being able to carry out the dechlorination also exists. This latter possibility may be associated with the uptake (availability) of the PCB or supply of a nutrient. It is also possible that during this lengthy process we enriched for a prototroph that no longer requires a component of the sediment in order to dechlorinate a PCB.

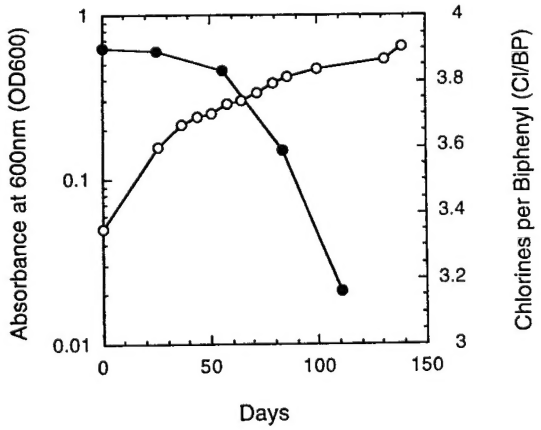

FIG. 4. OD (○) and chlorines-per-biphenyl (●) data from duplicate fifth-sequential-transfer cultures without sediment.

Sediment stimulation of *ortho* dechlorination. The above results demonstrate that *ortho* dechlorination is independent of the sediment. However, several results show the sediment to have a stimulatory effect. The first suggestion of this was observed with the decrease in the rate and extent of *ortho* dechlorination that accompanied the shift from *meta*-and-*ortho* to strictly *ortho* dechlorination (Fig. 1 and 2). This occurred after a primary culture had been transferred to a medium with far less sediment (5.0 to 0.1%, wt/vol [dry weight]). This change in activity could have been due to the decrease in the amount of sediment present. To examine this, a range of sediment concentrations was tested under the conditions described above. In order to be certain of the sediment concentration, the supernatant from the primary culture was transferred (10% [vol/vol]) into vessels containing E-Cl medium with the different amounts of BH sediment to be tested. After 4 months of incubation, transfers were made from these cultures into identical medium and the results of this second set of cultures are presented in Fig. 5. While dechlorinating activity could be maintained regardless of the sediment concentration, the lag preceding dechlorination increased to more than 100 days when the sediment concentration was lowered to 0.05% (dry wt). The cultures incubated with 1.0% (dry wt) sediment exhibited a higher rate of dechlorination and a shorter lag time than did those incubated with lesser amounts of sediment. Killed-cell controls (sterilized sediment cultures) exhibited no dechlorination. From a qualitative perspective, dechlorination did not change with sediment concentration and remained strictly *ortho*. Additional experiments with sediment-free cultures also demonstrated that the sediment could be stimulatory. Pre-dechlorination sediment-free cultures (in this case the

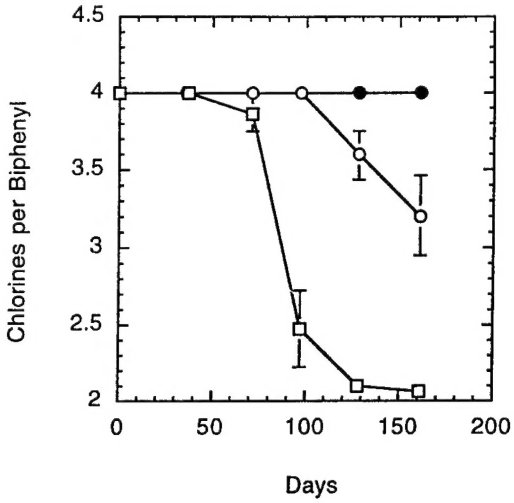

FIG. 6. Effect of sediment added to a pre-dechlorination sediment-free culture. The fourth-sequential-transfer sediment-free cultures (pre-dechlorination) were transferred to medium with (□) and without (○) 1.0% sterilized BH sediment. ●, killed-cell control. All data are for triplicate cultures. Error bars indicate standard deviations.

fourth sequential sediment-free transfer cultures before the onset of dechlorination) were transferred into E-Cl medium with and without 1.0% (dry weight) BH sediment (sterile). The pre-dechlorination transfer cultures with sediment showed a quicker recovery of dechlorination than did transfer cultures maintained without sediment (Fig. 6). Once again, no dechlorination was observed with killed-cell controls. This confirmed the existence of a factor(s) in the sediment that was stimulatory but not required for dechlorination.

The mode of action of the sediment stimulation of PCB dechlorination has not been determined. Humic acids (Aldrich Chemical Co., Milwaukee, Wis., and other sources) and anthraquinone-2,6-disulfonic acid (AQDS) have been shown to act as intermediate electron acceptors in the facilitation of biological Fe^{3+} reduction (4). Similarly, humic substances or AQDS might stimulate *ortho* PCB dechlorination. However, substitution of two different commercial humic acids at 0.1% (wt/vol [dry weight]) (Burlington Chemical Co., Long Island, N.Y., and Aldrich Chemical Co.) or 3 mM AQDS (Aldrich Chemical Co.) did not provide the same degree of stimulation of PCB dechlorination to the cultures as BH sediment (data not shown). In fact, the Aldrich humic acids and the AQDS completely inhibited dechlorination. The results from these experiments do not define the stimulatory role of the sediment, but they do demonstrate the utility of the sediment-free cultures in addressing such questions. Other possible roles for the BH sediment include stimulation due to additional carbon and energy or micronutrients, facilitation of the availability of the PCB to the microorganisms (this could also prevent toxicity due to PCBs), supply of a more suitable attachment site for microbial colonization, or supply of an extracellular catalytic intermediate similar to AQDS which may facilitate the dechlorination.

Concluding remarks. PCB dechlorination can occur in the absence of sediment, albeit more slowly than with sediment, indicating that whatever is contributed by the sediment is not essential or irreplaceable. We have now been able to sequentially transfer the *ortho*-dechlorinating culture eight times over a 33-month period in the minimal, sediment-free medium described here. Further, with the use of nearly identical enrichment procedures and patience, we have recently established enrichment cultures which actively *para* dechlorinate 2,3,4,5-CB in the absence of sediment. These cultures are incapable of dechlorinating 2,3,5,6-CB. We are still pursuing a *meta*-dechlorinating sediment-free enrichment culture.

Perhaps the most significant contribution of the findings presented here is that the sediment-free cultures offer opportunities to address questions of mechanism, cell structure, and identity that were not approachable in the past. For example, with the ability to make microscopic observation and determine OD, etc., questions concerning growth and whether it can be coupled to PCB dechlorination can now be more easily addressed. Isolation and characterization of the microorganisms present in these cultures can now proceed at a faster pace since the organisms are in a defined medium. We are taking advantage of this by investigating a broader group of electron donors and acceptors, individually, without interference from unknown substances in the sediment while monitoring the microbial population through molecular identification.

The development of the actively dechlorinating sediment-free cultures provides a unique opportunity for further experimentation concerning the identification of the stimulating factors in the sediment. In addition, the sediment-free cultures can act as a model system to investigate biochemical and geochemical mechanisms internal and external to the cell which may contribute to PCB dechlorination. Finally, studies of how to grow these organisms free of soil or sediment may lead to the ability to mass culture such organisms. This is needed in order to advance investigation of the biochemical mechanism of PCB dechlorination and could also be important for bioaugmentation studies of PCB-contaminated sediments.

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Anaerobic *ortho* Dechlorination of Polychlorinated Biphenyls by Estuarine Sediments from Baltimore Harbor

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Reductive dechlorination of the *ortho* moiety of polychlorinated biphenyls (PCBs) as well as of *meta* and *para* moieties is shown to occur in anaerobic enrichments of Baltimore Harbor sediments. These estuarine sediments *ortho* dechlorinated 2,3,5,6-chlorinated biphenyl (CB), 2,3,5-CB, and 2,3,6-CB in freshwater or estuarine media within a relatively short period of 25 to 44 days. *ortho* dechlorination developed within 77 days in marine medium. High levels of *ortho* dechlorination (>90%) occurred when harbor sediments were supplied with only 2,3,5-CB. Incubation with 2,3,4,5,6-CB or 2,3,4,5-CB resulted in the formation of the *ortho* dechlorination product 3,5-CB; however, *para* dechlorination of these congeners always preceded *ortho* chlorine removal. *ortho* dechlorination of PCBs is an exceedingly rare event that has not been reported previously for marine or estuarine conditions. The activity was reproducible and could be sustained through sequential transfers. In contrast, freshwater sediments incubated under the same conditions exhibited only *meta* and *para* dechlorinations. The results indicate that unique anaerobic dechlorinating activity is catalyzed by microorganisms in the estuarine sediments from Baltimore Harbor.

Because of their widespread use, stability, improper disposal, and potential toxicity, polychlorinated biphenyls (PCBs) remain a ubiquitous environmental concern (8, 12, 25, 26, 30) with an estimated 10 million tons (1 ton = ca. 906 kg), equivalent to one-third of the total worldwide production, having been released into the environment (9). PCBs are highly hydrophobic and strongly associate with organic carbon, clays, and silt that settle into the anaerobic regions of sediments. Estuarine and marine sediments are the ultimate global sinks for worldwide accumulation of PCBs sorbed to particulate material (13), and environmental transformations of PCBs in estuarine sediments have been documented (6, 15, 16). However, our understanding of the biological PCB transformation potential in marine and estuarine environments, particularly in anaerobic sediments where these compounds would be prevalent, is limited. The nature of estuarine and marine environments should make them particularly well suited for transformations of halogenated xenobiotics, including PCBs. Biogenically synthesized halogenated compounds, primarily in the form of brominated aliphatic and aromatic hydrocarbons, are ubiquitous among marine organisms ranging from eubacteria and algae to metazoans and hemichordates (10). Some species in the class *Rhodophyceae* are reported to accumulate organohalides at concentrations of up to 5% (dry weight) (11). Although brominated hydrocarbons are more prolific, many chlorinated substitutions have also been reported (10). Since these halogenated organic compounds do not continue to accumulate in the environment, it is likely that some processes must be transforming them.

In freshwater sediments, anaerobic reductive dechlorination of PCBs at all positions on the biphenyl ring has been reported (for a review of anaerobic dechlorination of PCBs, see reference 4). However, reductive *ortho* dechlorination under freshwater conditions has rarely been observed, and sustaining such activity is reported to be difficult (32, 33). Anaerobic PCB dechlorination has been shown to occur in estuarine sediments (6) and has been demonstrated in the laboratory under estuarine and marine conditions (2, 21), but the dechlorination is slow and not extensive. *ortho* dechlorination has not been reported to occur in estuarine or marine sediments. Herein, anaerobic PCB-dechlorinating activities of Baltimore Harbor (BH) sediments are characterized in marine, estuarine, and freshwater enrichment media. Dechlorination of the *ortho* positions in addition to *para* and *meta* positions of PCBs is described.

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MATERIALS AND METHODS

Sediment sample. Core samples (41 by 5 cm) of sediment were taken 8 m below the surface water in the Inner Harbor of Baltimore, Md. BH sediments were black in color, gelatinous in texture, and had a strong petroleum odor. The salinity of the water column immediately above the sediments was 10 ppt at the time of sampling. The lower 30 cm of sediment was immediately transferred to a glass container that had been purged with nitrogen. Sodium sulfide nonahydrate was added to a final concentration of 0.018% (wt/vol), and the vessel was sealed under nitrogen with a butyl rubber stopper. The sediment sample was stored at room temperature in the dark prior to use. Hudson River H7 sediments were graciously supplied by General Electric Co. (Schenectady, N.Y.) and stored as described above.

Culture conditions. All media in these experiments included modified basal medium (29) composed of the following components in grams per liter (final concentration) of demineralized water: Na_2CO_3, 3.0; Na_2HPO_4, 0.6; NH_4Cl, 0.5; cysteine-HCl · H_2O, 0.25; $Na_2S \cdot 9H_2O$, 0.25; resazurin, 0.001. In addition, 1% (vol/vol) each of vitamin and trace element solutions was added (34). Estuarine medium without sulfate (E-Cl medium) contained the following components in grams per liter (final concentration) of basal medium: NaCl, 8.4; $MgCl_2 \cdot 6H_2O$, 3.95; KCl, 0.27; $CaCl_2 \cdot 2H_2O$, 0.05. Estuarine salts medium with sulfate (E medium) contained the following components in grams per liter (final concentration) of basal medium: NaCl, 8.4; $MgSO_4 \cdot 7H_2O$, 4.44; KCl, 0.27; $CaCl_2 \cdot 2H_2O$, 0.05. Marine salts medium with sulfate (M medium) contained the following components in grams per liter (final concentration) of basal medium: NaCl, 23.38; $MgSO_4 \cdot 7H_2O$, 12.32; KCl, 0.76; $CaCl_2 \cdot 2H_2O$, 0.14. Sterile media were prepared anaerobically in an atmosphere that contained N_2-CO_2 (4:1) by a modification of the Hungate technique (3). All gasses were passed through a column of reduced copper turnings at 350°C to remove traces of O_2. Media (8 ml) were dispensed into culture tubes (16 by 160 mm) and sealed with Teflon-lined butyl stoppers (The West Co., Lionville, Pa.) secured by aluminum crimp collars (Bellco Glass, Inc., Vineland, N.J.).

BH sediments (20% [vol/vol]) were inoculated into media and incubated with individual PCB congeners at the following final concentrations in micromoles per

TABLE 1. Moles percent and recovery data for BH sediment incubated with 2,3,4,5-CB in E-Cl medium

Enrichment[a]	Day	Mol% (nmol of congener)[b]							% Total recovery (nmol)[c]
		2,3,4,5-CB	2,4,5-CB	2,3,5-CB	3,5-CB	2,4-CB/2,5-CB	3-CB	4-CB	
1	0	100 (92)	0 (0)	0 (0)	0 (0)	0/0 (0/0)	0 (0)	0 (0)	53 (92)
	30	58–56 (64)	4 (4)	12–11 (13)	19–18 (21)	8/11 (9/12)	0 (0)	0 (0)	64–66 (111–114)
	66	32–29 (36)	1 (1)	1 (1)	41–36 (45)	25/33 (28/41)	0 (0)	0 (0)	64–72 (111–124)
	128	11–9 (10)	<1 (<0.5)	<1 (<0.5)	53–46 (49)	36/45 (33/48)	0 (0)	0 (0)	53–62 (92–107)
2	0	100 (113)	0 (0)	0 (0)	0 (0)	0/0 (0/0)	0 (0)	0 (0)	65 (113)
	35	41–38 (39)	2 (2)	5 (5)	35–33 (34)	17/23 (16/24)	0 (0)	0 (0)	55–60 (96–104)
	35-R	77–74 (283)	<1 (1)	2 (7)	13 (49)	8/11 (28/42)	0 (0)	0 (0)	106–110 (368–382)
	54	50–45 (102)	2 (5)	2 (4)	23–21 (48)	23/31 (47/70)	0 (0)	0 (0)	60–66 (206–229)
	54-R	67–63 (264)	2–1 (6)	1 (5)	16–15 (63)	15/19 (55/81)	0 (0)	0 (0)	76–81 (393–419)
	71	50–45 (114)	4–3 (8)	1 (2)	15–14 (35)	26/34 (58/85)	2 (4)	2 (5)	44–49 (226–253)
	154	20–16 (57)	6–5 (16)	1 (2)	10–9 (30)	45/55 (131/192)	6–5 (16)	13–10 (36)	55–67 (288–349)

[a] Enrichment 1 (fatty acids replenished) received 173 μM 2,3,4,5-CB on day 0 and was replenished with the fatty acid mixture at each sampling (see Materials and Methods for concentrations). Enrichment 2 (PCB and fatty acids replenished) received 173 μM 2,3,4,5-CB on day 0 and was replenished with the same amount of PCB at times designated with R; fatty acids were added to enrichment 2 at each sampling.

[b] Data are in moles percent with the total nanomoles for each congener recovered from a 1-ml sediment sample shown in parentheses. Congeners 2,4-CB and 2,5-CB could not be chromatographically resolved. Therefore, values for 2,4-CB and 2,5-CB are calculated for both congeners as described in Materials and Methods. All other moles percent values are a range based on values calculated for 2,4-CB or 2,5-CB.

[c] Total recovery is expressed in a percentage with the nanomoles recovered shown in parentheses (range once again dependent upon 2,4-CB or 2,5-CB).

liter: monochlorobiphenyls, 266; dichlorobiphenyls, 225; trichlorobiphenyls, 195; tetrachlorobiphenyls, 173; pentachlorobiphenyls, 154. Because of their low solubility in water, the congeners were solubilized in acetone before addition to the sediments. The final concentration of acetone was 0.1% (vol/vol). Sodium acetate, propionate, and butyrate were added to a final concentration of 2.5 mM each. Cultures were incubated at 30°C in the dark. Sterile controls (sterilized sediments) were autoclaved at 121°C for 3 h.

Sterilized controls with 2,3,4,5-, 2,3,5,6-, 2,3,6-, or 2,3,5-chlorinated biphenyl (CB) in estuarine medium without sulfate showed no activity for up to 128 to 154 days. The percent recoveries of total PCBs from all of these controls (calculated from seven incubations) were 53 ± 14 at day 0, 60 ± 17 at day 30, 50 ± 12 at day 60, 43 ± 11 at day 91, and 42 ± 10 at day 128. Similar recoveries were found in live cultures (see Tables 1 to 4).

Culture sampling and sample preparation. Replicate enrichments were sampled once at each time point. Cultures were sampled under O_2-free N_2, and samples were extracted as described previously (17, 22). Briefly, culture tubes were shaken, and a 1-ml slurry sample was immediately removed from the bottom of the tube with the reverse end of a 2-ml glass pipette. PCBs in the sample were extracted by shaking overnight with 10 ml of ethyl acetate in a 15-ml glass vial sealed with a Teflon-lined screw cap. After extraction, the organic phase was passed through a Florisil-copper column.

PCB analysis. PCB congeners were identified with a Hewlett-Packard 5890A gas chromatograph equipped with an electron capture detector (GC-ECD) and a RTX-1 capillary column (0.25 mm by 30 m; Restek Corp., Bellefonte, Pa.) as described by May et al. (17). PCB congeners were identified by retention time, and the relative molar distribution of congeners was determined from standard curves for individual congeners. Sixteen-point standard curves were individually developed for each congener. Congeners 2,4- and 2,5-CB could not be separated by the GC methods employed. In cases where 2,4- or 2,5-CB could be present, two calculations were made, one assuming all of the product as 2,4-CB and the other assuming all of the products as 2,5-CB. Congener 3-CB would shoulder onto 4-CB when both were present. A tangential integration was used to quantitate both. Identification of PCBs and biphenyl was confirmed by GC-mass spectrometry (GC-MS) with a Hewlett-Packard 5970 mass selective detector coupled to a Hewlett-Packard 5890A GC. The GC conditions were identical to those described above. In addition to the retention times expressed in the total ion chromatographs, mono-, di-, and trichlorobiphenyls were identified by their respective molecular ions (*m/z* 188, 222, and 256) and fragmentation patterns. Biphenyls, monochlorobiphenyls, and dichlorobiphenyls were assayed by selective ion monitoring at *m/z* values of 154, 188, and 222. The minimum detection limit for biphenyl was <10 pg for a 1-μl injection.

Chemicals. All PCBs were obtained at >99% purity from Accustandard Inc., New Haven, Conn. High-performance liquid chromatography (HPLC)-grade ethyl acetate was purchased from Fisher Scientific, Pittsburgh, Pa. All other chemicals were of reagent grade.

RESULTS

***ortho* dechlorination in estuarine media.** Reductive dechlorination of *ortho*-positioned chlorine atoms was observed when BH sediment was incubated anaerobically in estuarine medium without sulfate. GC-ECD analysis showed dechlorination of 2,3,4,5-CB and subsequent formation of the *ortho* dechlorination product 3,5-CB within 25 to 35 days. Identification of 3,5-CB was based on the retention time of 3,5-CB and the fact that no other dichlorinated biphenyl from the dechlorination of 2,3,4,5-CB has a retention time near that of 3,5-CB. In addition, analysis on a GC-MS confirmed that the peak with the same retention time as 3,5-CB had the same molecular ion (*m/z* 222) and fragmentation pattern as 3,5-CB.

The moles percent distribution of PCB congeners was monitored in two separate BH sediment enrichments (Table 1). Each enrichment culture was incubated with 2,3,4,5-CB, but only one of the cultures was replenished with 2,3,4,5-CB. Congener 3,4,5-CB was not detected in either enrichment. Therefore, *para* dechlorination likely preceded *ortho* dechlorination and 2,3,5-CB then became the *ortho* dechlorination substrate. In the non-PCB-replenished enrichment (enrichment 1), approximately 90% of the 2,3,4,5-CB was transformed within 128 days. Congeners 2,3,5-CB and 2,4,5-CB appeared to be transient, with nearly half of the parent congener (2,3,4,5-CB) eventually being converted to 3,5-CB. No monochlorobiphenyl was detected in the non-PCB-replenished enrichment. In the PCB-replenished culture (enrichment 2), there was significant production of 3,5-CB during the first 35 days. These data suggest that *ortho* dechlorination (3,5-CB production) was sustained. However, this interpretation is inconclusive because it was difficult later to assess the production of the 3,5-CB due to its conversion to 3-CB.

The accumulation of 4-CB late in the incubation of the enrichment 2 culture (Table 1) is indicative of a second *ortho* dechlorination that likely results from 2,4-CB. A single separate incubation of BH sediment with 2,3,4-CB also resulted in *ortho* dechlorination with the production of both 2,4-CB and 4-CB (data not shown). However, separate incubations of BH sediment with 2,4-CB and 2,4,5-CB for 154 days did not result in the formation of 4-CB. Congener 2-CB was never detected in any of these enrichments. Selective ion monitoring at *m/z* 154 by GC-MS did not detect biphenyl in any of the enrichments, indicating that complete dechlorination also did not occur. No activity was observed in sterile controls after 154 days.

TABLE 2. Moles percent and recovery data for BH sediment incubated with 2,3,5-CB in E-Cl medium

Enrichment[a]	Day	Mol% (nmol of congener)[b]				% Total recovery[c]
		2,3,5-CB	3,5-CB	2,5-CB	3-CB	
1	0	100 (141)	0 (0)	0 (0)	0 (0)	72 (141)
	30	49 (75)	51 (79)	0 (0)	0 (0)	79 (154)
	66	2 (3)	98 (125)	0 (0)	0 (0)	66 (128)
	128	0 (0)	9 (10)	1 (1)	90 (97)	55 (108)
2	0	100 (168)	0 (0)	0 (0)	0 (0)	86 (168)
	35	21 (33)	79 (123)	0 (0)	0 (0)	80 (156)
	35-R	51 (212)	49 (203)	0 (0)	0 (0)	106 (415)
	54	15 (30)	58 (117)	2 (5)	24 (49)	52 (201)
	54-R	62 (388)	27 (171)	1 (6)	10 (64)	107 (629)
	71	14 (50)	35 (120)	30 (105)	20 (70)	59 (345)
	154	9 (32)	12 (42)	60 (205)	18 (62)	58 (341)

[a] Enrichment 1 (fatty acids replenished) received 195 μM 2,3,5-CB on day 0 and was replenished with the fatty acid mixture at each sampling (see Materials and Methods for concentrations). Enrichment 2 (PCB and fatty acids replenished) received 195 μM 2,3,5-CB on day 0 and was replenished with the same amount of PCB at times designated with R; fatty acids were added to enrichment 2 at each sampling. Replenishment was identical to that described in Table 1.

[b] Data are in moles percent with the total nanomoles for each congener recovered from a 1-ml sediment sample shown in parentheses.

[c] Total recovery is expressed as a percentage with the nanomoles recovered shown in parentheses.

The data from dechlorination of 2,3,4,5-CB suggest that 2,3,5-CB is a substrate for *ortho* dechlorination. Moles percent analyses of two separate enrichments show that *ortho* dechlorination of 2,3,5-CB is heavily favored in the reductive dechlorination of this congener (Table 2). *ortho* dechlorination was sustained in an enrichment replenished with 2,3,5-CB (enrichment 2). Only when an enrichment was replenished did *meta* dechlorination of 2,3,5-CB to 2,5-CB develop. This was confirmed in enrichment 1 by the addition of 2,3,5-CB after 128 days (data not shown). Congener 3-CB accumulated in both enrichments after extended incubation. Selective ion monitoring for *m/z* 188 by GC-MS confirmed the presence of a monochlorinated biphenyl that elutes at the retention time of 3-CB. The formation of 3-CB may have resulted from *meta* dechlorination of 3,5-CB or *ortho* dechlorination of 2,5-CB. However, separate incubations of BH sediment with 2,5-CB showed no dechlorination after 154 days, and incubations with 3,5 CB resulted in the formation of 3-CB. Biphenyl was not detected by selective ion monitoring. These observations suggest that the formation of 3-CB from 2,3,5-CB, and possibly from 2,3,4,5-CB, results from the sequential dechlorination of the *ortho* moiety followed by *meta* dechlorination. The accumulation of high amounts of 2,5-CB in enrichment 2, after 2,3,5-CB had been depleted to low levels and high amounts of 3,5-CB had previously accumulated, is an anomaly that cannot be explained at this time. Total extraction of the entire enrichment culture (two ethyl acetate and two hexane acetone extractions) after 328 days of incubation recovered 42% of the added PCBs and the moles percent distribution (2,3,5-CB, 2; 3,5-CB, 14; 2,5-CB, 62; 3-CB, 22) remained relatively the same as that at 154 days.

Expanding the survey to include incubations of BH sediments with other individual PCB congeners resulted in the discovery of two other *ortho* dechlorinations. The tetrachlorobiphenyl 2,3,5,6-CB was both *meta* and *ortho* dechlorinated. Table 3 shows the moles percent distributions from two separate enrichment cultures. The accumulation of *ortho* dechlorination products 2,3,5- and 3,5-CB was dominant early on in the non-PCB-replenished enrichment. Congener 3-CB was the major product at day 128 in this enrichment culture. Such products were also present in the PCB-replenished enrichment, but more of the *meta* dechlorination products 2,3,6- and 2,6-CB accumulated. The formation of 2,5-CB could have been due to *ortho* or *meta* dechlorination. The data from Table 2 shows sustained *ortho* activity. However, this is once again difficult to assess later on because of the production of 2,5- and 3-CB.

Another congener observed to be *ortho* dechlorinated was 2,3,6-CB. Moles percent analysis of BH sediment incubated with only 2,3,6-CB showed that the ratio of 2,6-CB to 2,5-CB was nearly 3:1 in duplicate enrichments (Table 4). Replenishing the cultures with 2,3,6-CB had no effect on the ratio of 2,6-CB to 2,5-CB. However, *ortho* dechlorination was sustained in the replenished enrichments. Although the amount of 2,5-CB produced is significant, it does not appear that *ortho* dechlorination of 2,3,6-CB is as extensive as that of 2,3,5-CB. A small amount of 2,3-CB which represents another *ortho* dechlorination, that of position 6 of 2,3,6-CB, also appeared in both enrichments.

TABLE 3. Moles percent and recovery data for BH sediment incubated with 2,3,5,6-CB in E-Cl medium

Enrichment[a]	Day	Mol% (nmol of congener)[b]							% Total recovery (nmol)[c]
		2,3,5,6-CB	2,3,6-CB	2,3,5-CB	3,5-CB	2,5-CB	2,6-CB	3-CB	
1	0	100 (98)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	57 (98)
	30	99 (112)	<1 (<0.5)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	65 (113)
	66	9 (15)	1 (1)	3 (4)	56 (88)	18 (28)	4 (7)	9 (15)	91 (158)
	128	1 (1)	<1 (<0.5)	1 (1)	6 (6)	22 (24)	6 (6)	65 (71)	63 (109)
2	0	100 (129)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	75 (129)
	35	89 (109)	1 (2)	7 (8)	2 (2)	1 (1)	0 (0)	0 (0)	71 (122)
	35-R	97 (277)	<1 (1)	2 (6)	1 (2)	0 (0)	0 (0)	0 (0)	83 (286)
	54	32 (77)	13 (31)	2 (5)	3 (7)	29 (69)	22 (52)	0 (0)	70 (241)
	54-R	63 (335)	8 (41)	1 (5)	2 (11)	15 (77)	12 (62)	0 (0)	102 (531)
	71	22 (68)	26 (80)	3 (10)	4 (11)	29 (88)	16 (49)	0 (0)	59 (306)
	154	18 (57)	14 (44)	2 (8)	5 (15)	31 (102)	26 (85)	4 (14)	63 (325)

[a] Enrichment 1 (fatty acids replenished) received 173 μM 2,3,5,6-CB on day 0 and was replenished with the fatty acid mixture at each sampling (see Materials and Methods for concentrations). Enrichment 2 (PCB and fatty acids replenished) received 173 μM 2,3,5,6-CB on day 0 and was replenished with the same amount of PCB at times designated with R; fatty acids were added to enrichment 2 at each sampling. Replenishment was identical to that described in Table 1.

[b] Data are in moles percent with the total nanomoles for each congener recovered from a 1-ml sediment sample shown in parentheses.

[c] Total recovery is expressed as a percentage with the nanomoles recovered shown in parentheses.

TABLE 4. Moles percent and recovery data for BH sediment incubated with 2,3,6-CB in E-Cl medium

Enrichment[a]	Day	Mol% (nmol of congener)[b]					% Total recovery (nmol)[c]
		2,3,6-CB	2,3-CB	2,5-CB	2,6-CB	3-CB	
1	0	100 (176)	0 (0)	0 (0)	0 (0)	0 (0)	90 (176)
	30	97 (126)	0 (0)	1 (1)	2 (3)	0 (0)	67 (130)
	66	25 (38)	<1 (<0.5)	16 (25)	58 (89)	0 (0)	78 (152)
	128	5 (6)	<1 (<0.5)	20 (27)	75 (99)	0 (0)	68 (132)
2	0	100 (83)	0 (0)	0 (0)	0 (0)	0 (0)	43 (83)
	35	72 (59)	0 (0)	7 (6)	21 (17)	0 (0)	42 (82)
	35-R	92 (263)	0 (0)	2 (6)	6 (18)	0 (0)	74 (287)
	54	14 (49)	0 (0)	23 (81)	63 (225)	0 (0)	91 (355)
	54-R	54 (407)	0 (0)	11 (85)	34 (255)	0 (0)	128 (747)
	71	21 (70)	0 (0)	19 (65)	60 (199)	0 (0)	57 (334)
	154	12 (17)	<1 (<0.5)	18 (26)	71 (103)	0 (0)	25 (146)

[a] Enrichment 1 (fatty acids replenished) received 195 μM 2,3,6-CB on day 0 and was replenished with the fatty acid mixture at each sampling (see Materials and Methods for concentrations). Enrichment 2 (PCB and fatty acids replenished) received 195 μM 2,3,6-CB on day 0 and was replenished with the same amount of PCB at times designated with R; fatty acids were added to enrichment 2 at each sampling. Replenishment was identical to that described in Table 1.

[b] Data are in moles percent with the total nanomoles for each congener recovered from a 1-ml sediment sample shown in parentheses. Some of the 2,3,6-CB used in these experiments was contaminated with 0.4% 2,3,4,6-CB. All of the 2,3,4,6-CB was transformed and a corresponding amount of 2,4,6-CB accumulated. These two congeners are not included in the data shown.

[c] Total recovery is expressed as a percentage with the nanomoles recovered shown in parentheses.

Congener 2,3,4,5,6-CB was also dechlorinated when incubated with BH sediments. Congener 2,3,4,5-CB was never observed in cultures incubated with 2,3,4,5,6-CB. Therefore, similar to the dechlorination of 2,3,4,5-CB, *meta* and/or *para* dechlorinations must precede *ortho* dechlorination of this pentachlorinated biphenyl. Significant amounts of 2,3,5-CB and 3,5-CB from 2,3,4,5,6-CB were detected over time, but 2,4,6-CB was the most prevalent product. Replicate enrichments gave similar results.

Several other congeners were tested individually in separate enrichments of BH sediment, but none were *ortho* dechlorinated. The following congeners were not dechlorinated at all: 2,2',6,6'-CB, 2,4,6-CB, 2,2'-CB, 2,4-CB, 2,5-CB, 2,6-CB, 2-CB, 3-CB, and 4-CB (minimum of 145 days of incubation). No loss of a monochlorinated biphenyl was ever observed, and biphenyl was not detected by GC-MS in the enrichments incubated with monochlorobiphenyls. The following transformations were observed: 2,4,5-CB to 2,4- or 2,5-CB; 3,4,5-CB to 3,4-CB, 3,5-CB, and 3-CB; 2,3-CB to 2-CB; 3,4-CB to 3-CB; and 3,5-CB to 3-CB. Although 2,3,5-CB and 2,3,6-CB were *ortho* dechlorinated, it is interesting to note that 2,4,5-CB, 2,4,6-CB, and all of the *ortho*-chlorinated biphenyls tested, at least in individual incubations, were not *ortho* dechlorinated. These results suggest that *ortho* dechlorination occurs when the biphenyl ring is sufficiently chlorinated and contains a *meta* chlorine adjacent to the *ortho* chlorine.

Supernatants from several of the enrichments described above have been serially transferred in fresh E-Cl medium plus sterile BH sediment, coal-based humic acids, or Hudson River Spier Falls sediment (non-PCB contaminated). Dechlorination has been observed as early as 7 days in these transferred cultures, and *ortho* dechlorination develops within 21 days. Activity in these transfers has been observed with 0.05 to 1.0% (wt/vol [dry weight]) sediment in the medium.

***ortho* dechlorination in other estuarine, marine, and nonmarine media.** Sulfate is prevalent in marine and estuarine environments at concentrations that are reported to inhibit dechlorination of PCBs in freshwater sediments (2, 6, 31). However, PCB dechlorination has been shown to occur anaerobically with estuarine sediments in the presence of high concentrations of sulfate (21). To determine the effects of sulfate on PCB dechlorination in BH sediments, enrichments were incubated with 2,3,4,5-CB in estuarine (E) and marine (M) media that contained 18 and 50 mM $MgSO_4 \cdot 6H_2O$, respectively. For enrichments in both of these media, the moles percent distribution of congeners was very similar to that observed with the E-Cl medium, but activity in the M medium lagged (no dechlorination at 44 days, dechlorination including *ortho* at 77 days). Marine medium without sulfate ($MgCl_2 \cdot 6H_2O$ substituted for $MgSO_4 \cdot 6H_2O$) also supported *ortho* dechlorination but with less of a lag than enrichments with sulfate in the medium. These results demonstrate that anaerobic PCB dechlorination including *ortho* dechlorination develops in BH sediments inoculated into media containing relatively high sulfate and solute concentrations associated with estuarine and marine conditions. It is possible that the sulfate is consumed before dechlorination sets in. However, since sulfate was not monitored in these enrichments, the effect of sulfate on PCB dechlorination is inconclusive at this time.

Reduced anaerobic mineral medium (RAMM) (27) has been used with sediments from freshwater sites such as the upper Hudson River (1, 5, 19, 23). *ortho* dechlorination has never been reported with Hudson River sediments in RAMM. Our incubations of Hudson River H7 sediment (supplied by General Electric) with 2,3,4,5-CB in RAMM did result in *meta* and *para* dechlorination within 22 days, and no *ortho* dechlorination was observed over a period of 124 days. No *ortho* dechlorination was observed with Hudson River sediment in E, M, E-Cl, or M-Cl medium. Incubations of BH sediment in RAMM with 2,3,4,5-CB did result in *ortho* dechlorination. Once again, the transformations observed were qualitatively similar to those observed with E-Cl medium. However, the BH enrichments showed high levels of 2,3,5-CB early during incubation followed by high accumulations of 3,5-CB with no production of 3-CB. Congener 2,4,5-CB was produced only in trace amounts. These activities suggest a shift from dechlorination of the *meta* moiety to that of the *para* and *ortho* moieties when the BH sediments are incubated in RAMM, a nonmarine medium.

DISCUSSION

A review of all of the experiments presented here demonstrates that a rare and unique type of anaerobic PCB dechlorination (*ortho*) arises rapidly in enrichments containing BH sediment. The major *ortho* dechlorination pathways observed are summarized in Fig. 1. Since BH is part of the Chesapeake Bay, which is an extensive drainage basin, there is likely a gradation of freshwater, estuarine, and marine microbial communities along the length of the bay. Sediments in BH may contain components of the three communities. While *ortho*-dechlorinating activity might be attributed to populations of estuarine and marine microorganisms, wide differences in reductively dechlorinating populations have been reported among sites of close proximity in the St. Lawrence River. These differences have been attributed to sediment characteristics (28). It is therefore possible that undefined conditions in BH select for PCB-dechlorinating populations that include *ortho* dechlorination. PCBs have been reported to be associated with the particulate fraction of the water column of the Chesapeake Bay (14), and PCB contamination of BH sediment has been documented (20). PCBs were not detected in BH sediments by

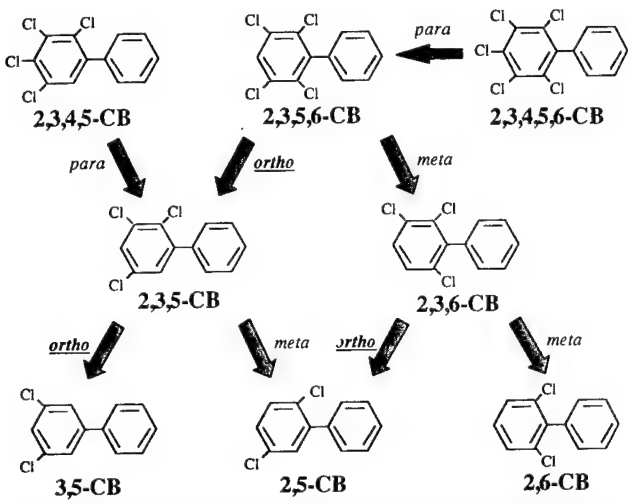

FIG. 1. *ortho* dechlorination pathways observed in this study. Not all dechlorinations are represented. The figure depicts the most prevalent *ortho* reactions and the necessary precursor reactions. Predominance of pathway or step is not indicated since this may vary with the PCB mixture and environmental conditions.

methods described in this study. However, past contamination of the harbor with PCBs or other chlorinated organics may have promoted in situ selection of dechlorinating organisms.

The removal of *ortho* chlorines from PCBs has not been demonstrated previously with estuarine or marine sediments. In addition, reports on *ortho* dechlorination with anaerobes from any environment have been infrequent. The activity has always required several months to develop and has been difficult to repeat or maintain. The best-documented report of *ortho* dechlorination was published by Van Dort and Bedard (32) and showed *ortho* dechlorination of 2,3,5,6-CB to 2,5-CB, via either 2,3,6-CB or 2,3,5-CB, in one freshwater sediment culture from Woods Pond (Lenox, Mass.) after 21 weeks of incubation. After 37 weeks, 19.4% of the PCB had been converted to 2,5-CB and 58.2% had been converted to 2,6-CB. The balance of PCB consisted of 2,3,6-CB, a trace of 2,3,5-CB, and residual 2,3,5,6-CB. Congener 2,3,5-CB never accumulated to high levels, and 3,5-CB was not detected. The *ortho*-dechlorinating activity by the Woods Pond culture ceased after 28 weeks and was subsequently followed by *meta* dechlorination. The *ortho*-dechlorinating activity did not return, and the authors attributed the loss of *ortho* activity to a change in dechlorinating populations. Williams (33) also reported that a culture from Woods Pond and one sediment culture from Silver Lake (Pittsfield, Mass.) *ortho* dechlorinated 2,4,6-CB to 2,4-CB and 4-CB after 24 weeks and more than 1 year of incubation, respectively. Sustainability was not addressed in this study. Montgomery and Vogel (18) reported that 2,3,5,6-CB was *ortho* dechlorinated to 2,3,5-CB and 3,5-CB over a 14-month period by sediment cultures under anaerobic phototrophic conditions. The investigators reported dechlorination in the dark to be nonexistent or negligible. Unfortunately, the authors did not prepare and monitor a killed-cell (sterilized-sediment) control, which would have been useful in interpreting the results since PCBs have been shown to be photochemically dechlorinated, particularly at the *ortho* position (7, 24). All of the experiments reported here involved incubation in the dark.

In contrast with *ortho*-dechlorinating activity observed with freshwater sediments, the *ortho* dechlorination observed with BH sediments is different in that 2,3,5-CB and 3,5-CB are very prevalent products in BH sediments when 2,3,4,5-CB, 2,3,5,6-CB, or 2,3,5-CB is present. Another congener specificity difference is that 2,4,6-CB was not dechlorinated in BH sediments, although this congener may require a lengthier incubation. In addition, the *ortho* dechlorination observed in BH sediment develops relatively quickly and can be very extensive with acclimation times of less than 1 month and greater than 90% *ortho* transformation of 2,3,5-CB to 3,5-CB in some enrichments. The *ortho* dechlorination is also readily maintained and reproduced. The enrichments described here were inoculated with sediments collected on 19 July 1992. *ortho*-dechlorinating enrichments have also been developed with fresh BH sediment collected from the same site on 11 July 1995. Activity has been maintained for more than 6 months by replenishment with media and 2,3,4,5-CB, and *ortho* dechlorination has been observed in five different media, including those with high solute concentrations. Transfer of the activity to fresh media has also been successful, with serial transfers demonstrating *ortho* dechlorination within 21 days.

The *ortho* dechlorination by BH sediments also appears to be broad in that a variety of congeners, including tetra-, tri-, and dichlorobiphenyls, are attacked (*ortho* dechlorination of dichlorobiphenyls appeared to have occurred only when tetra- or trichlorobiphenyls were present). The lower levels of dichlorobiphenyls and 3-CB in enrichments replenished with 2,3,5,6-CB (Table 3) suggest that the tetrachlorobiphenyl is more readily *ortho* dechlorination than lesser-chlorinated congeners and perhaps can act as a more attractive electron acceptor for an *ortho* PCB-dechlorinating anaerobe. This is consistent with previous observations that more extensively chlorinated PCBs are more readily *meta* and *para* dechlorinated in anaerobic freshwater sediment enrichments (4).

The demonstration of *ortho*-dechlorinating activity with BH sediments suggests that previously undescribed estuarine or marine anaerobic microorganisms present in these sediments are capable of unique activity or that environmental conditions enhance a biological activity rarely observed. Other marine and estuarine sites are being investigated by the methods described here to determine the prevalence of *ortho* dechlorination. Environmental conditions such as solute concentration, carbon sources, pH, and sediment, etc., are also under investigation. Additionally, the dechlorination of commercial mixtures of PCBs (Aroclors) is being examined. BH sediments amended with Aroclor 1242 (400 ppm) or Aroclor 1242 plus 2,3,4,5-CB (172.5 μM) have been analyzed after 2 months of incubation. Thus far, the 2,3,4,5-CB was *ortho* dechlorinated to 3,5-CB within 1 month, indicating that the Aroclor mixture does not inhibit *ortho* dechlorination of this congener. Congeners belonging to Aroclor 1242 were transformed after 2 months of incubation. A complete analysis of Aroclor dechlorination requires further incubation time and PCB analysis. In addition, the investigations presented here deal primarily with the dechlorination of congeners that are chlorinated on only one ring. Incubations of BH sediments with PCBs chlorinated on both rings, especially *ortho*-substituted and more heavily chlorinated congeners, is needed to further define the dechlorination potential of the anaerobic microorganisms in these sediments. The results presented in this study suggest that the development of *ortho* dechlorination in conjunction with activity specific for other chlorine substitutions could be combined for more extensive reductive dechlorination of PCBs in anaerobic environments.

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Characterization of a Defined 2,3,5,6-Tetrachlorobiphenyl-*ortho*-Dechlorinating Microbial Community by Comparative Sequence Analysis of Genes Coding for 16S rRNA

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Defined microbial communities were developed by combining selective enrichment with molecular monitoring of total community genes coding for 16S rRNAs (16S rDNAs) to identify potential polychlorinated biphenyl (PCB)-dechlorinating anaerobes that *ortho* dechlorinate 2,3,5,6-tetrachlorobiphenyl. In enrichment cultures that contained a defined estuarine medium, three fatty acids, and sterile sediment, a *Clostridium* sp. was predominant in the absence of added PCB, but undescribed species in the δ subgroup of the class *Proteobacteria*, the low-G+C gram-positive subgroup, the *Thermotogales* subgroup, and a single species with sequence similarity to the deeply branching species *Dehalococcoides ethenogenes* were more predominant during active dechlorination of the PCB. Species with high sequence similarities to *Methanomicrobiales* and *Methanosarcinales* archaeal subgroups were predominant in both dechlorinating and nondechlorinating enrichment cultures. Deletion of sediment from PCB-dechlorinating enrichment cultures reduced the rate of dechlorination and the diversity of the community. Substitution of sodium acetate for the mixture of three fatty acids increased the rate of dechlorination, further reduced the community diversity, and caused a shift in the predominant species that included restriction fragment length polymorphism patterns not previously detected. Although PCB-dechlorinating cultures were methanogenic, inhibition of methanogenesis and elimination of the archaeal community by addition of bromoethanesulfonic acid only slightly inhibited dechlorination, indicating that the archaea were not required for *ortho* dechlorination of the congener. Deletion of *Clostridium* spp. from the community profile by addition of vancomycin only slightly reduced dechlorination. However, addition of sodium molybdate, an inhibitor of sulfate reduction, inhibited dechlorination and deleted selected species from the community profiles of the class *Bacteria*. With the exception of one 16S rDNA sequence that had the highest sequence similarity to the obligate perchloroethylene-dechlorinating *Dehalococcoides*, the 16S rDNA sequences associated with PCB *ortho* dechlorination had high sequence similarities to the δ, low-G+C gram-positive, and *Thermotogales* subgroups, which all include sulfur-, sulfate-, and/or iron(III)-respiring bacterial species.

The extensive industrial use of polychlorinated biphenyls (PCBs) during the 20th century has resulted in the release of an estimated several million pounds of PCBs into the environment (2). Due to the hydrophobicity and chemical stability of these compounds, PCBs ultimately accumulate in subsurface anaerobic sediments, where reductive dechlorination by anaerobic microorganisms is proposed to be an essential step in PCB degradation and detoxification (6). Although anaerobic reductive dechlorination has been documented in the environment and in the laboratory, attempts to identify and isolate anaerobic PCB-dechlorinating microbes by classical enrichment and isolation techniques have been unsuccessful (for a review, see reference 2). Isolation of anaerobic PCB-dechlorinating microbes has been hindered in part by the inability to maintain and sequentially transfer dechlorinating consortia in defined medium. May et al. (24) were the first to demonstrate that single colonies could be obtained by plating highly enriched PCB-dechlorinating enrichment cultures on agar-solidified media. Although two of the colonies exhibited *para* dechlorination activity when transferred back to liquid enrichment medium, the colonies contained a mixed community of microorganisms and dechlorination required the addition of sediment to the medium. More recently, highly enriched PCB-*ortho*-dechlorinating enrichment cultures were developed from Baltimore Harbor sediments in minimal media that contained sediments and a single congener (3) or Aroclor 1260 (37). These were the first confirmed reports of sustained *ortho* dechlorination of PCBs throughout sequential transfers in medium with estuarine sediments. Finally, Cutter et al. demonstrated that a consortium of PCB-*ortho*-dechlorinating anaerobes from Baltimore Harbor could be sequentially transferred and maintained in minimal medium without the addition of sterile sediment (9). With the ability to maintain PCB dechlorination in a completely defined medium, highly enriched PCB-dechlorinating consortia could be developed by sequential transfers in medium that contained the minimal growth requirements for dechlorinating species.

The current study identifies putative PCB-dechlorinating anaerobes in *ortho*-dechlorinating enrichment cultures by a comprehensive approach that combines traditional selective enrichment techniques with molecular monitoring (SEMM). Microbial consortia enriched for PCB *ortho* dechlorination in minimal medium were analyzed by comparative sequence analysis of genes coding for 16S rRNA (16S rDNA) amplified from total community DNAs. Protocols were developed for chro-

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mosomal DNA extraction from sediment, 16S rDNA amplification by PCR, cloning of partial 16S rDNA PCR fragments, screening by restriction fragment length polymorphism (RFLP) analysis, and DNA sequencing for comparative sequence analysis. By utilizing these techniques, shifts in the microbial community were monitored as the cultures were further enriched for PCB-dechlorinating anaerobes by elimination of undefined medium components (i.e., sediment), changes in carbon source, and addition of selective physiological inhibitors. The results presented herein demonstrate the applicability of the SEMM approach for the selection and monitoring of highly defined PCB-dechlorinating microbial consortia.

MATERIALS AND METHODS

Enrichment cultures. Enrichment cultures were initiated as described previously (9). Briefly, sediment samples collected from the Northwest Branch of Baltimore Harbor, Baltimore, Md. (39°16.8′N, 76°36.1′W), were used to inoculate sterile, anaerobic estuarine salts medium that did not contain added sulfate to a final concentration of 5% (dry wt/vol). Where indicated, sodium acetate, alone or with sodium propionate and butyrate, was added to a final concentration of 2.5 mM (each). The congener 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB; AccuStandard, Inc., New Haven, Conn.) was solubilized in acetone and added to a final concentration of 173 μM. For the inhibitor studies, bromoethanesulfonic acid (BES), vancomycin, and sodium molybdate were dissolved in deionized water, filter sterilized, and added to final concentrations of 3 mM, 100 μg/ml, and 20 mM, respectively. All cultures were incubated in the dark at 30°C. PCBs were extracted and analyzed by gas chromatography coupled with an electron capture detector using a 16-point standard curve for each congener as described previously (3).

Extraction of genomic DNA. The methods described herein for the phylogenetic analysis of the enrichment cultures are slightly modified from those described previously (13). Depending upon the culture turbidity, between 1 and 10 ml of culture was anaerobically withdrawn and utilized for extraction of bulk genomic DNA (final yield, greater than 100 ng as estimated by visualization on an agarose gel stained with ethidium bromide). The culture sample was centrifuged, and the cell and sediment pellet was resuspended in 250 μl of sterile TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). The resuspended pellet was added to a 2.2-ml screw-cap conical tube that contained 2.5 g of autoclaved zirconia-silica beads (0.1 mm), and 250 μl each of sodium phosphate buffer (0.1 M, pH 8.0) and TS-SDS buffer (0.1 M NaCl, 0.5 M Tris [pH 8.0], 10% [wt/vol] sodium dodecyl sulfate). The sample was cooled on ice for 10 min and then homogenized for 5 min with a Mini-Bead Beater (Biospec, Bartlesville, Okla.) at 4°C to lyse cells. Debris was removed by centrifugation for 5 min at 14,000 × *g*. Crude DNA in the supernatant was purified twice with equal volumes of trissaturated phenol and chloroform-isoamyl alcohol (24:1), followed by extraction with an equal volume of chloroform. Approximately 200 μl of Phase-Lock gel (5 Prime-3 Prime, Inc., Boulder, Colo.) was utilized to promote separation of the phases and allow easier visualization of the interface. The decanted aqueous phase was diluted to 1 ml with sterile deionized water. Humic acids, which inhibit PCR (32, 34), were extracted from nucleic acids by addition of 0.125 g of insoluble polyvinylpolypyrrolidone (Sigma, St. Louis, Mo.) to the 1 ml of diluted crude DNA extract (17, 30). The polyvinylpolypyrrolidone was removed by centrifugation for 5 min at 14,000 × *g*, and the chromosomal DNA was recovered by precipitation with an equal volume of isopropanol at −20°C. The DNA was pelleted by centrifugation, and then the pellet was washed with 70% ethanol and centrifuged again at high speed. The supernatant was discarded, and the DNA was dried under vacuum for 5 min. Further removal of humic acids was achieved by electrophoresis of the DNA extract in a 1.3% low-melting-point agarose gel (Fisher Scientific, Fairlawn, N.J.) containing 2% soluble polyvinylpyrrolidone (40). The chromosomal DNA band was excised from the gel and recovered with a Promega Wizard PCR Prep Kit (Promega, Madison, Wis.) in accordance with the manufacturer's instructions.

PCR amplification and cloning. PCR was utilized to amplify bacterial and archaeal 16S rDNAs from the mixed community of genomic DNAs. Universal primers 519F (5′-CAG CA/CG CCG CGG TAA TA/TC-3′) and 1406R (5′-ACG GGC GGT GTG TA/GC-3′) were utilized for the amplification of bacterial 16S rDNAs (21). Archaeal 16S rDNAs were amplified with specific archaeal primers 21F (5′-TTC CGG TTG ATC CYG CCG GA-3′) and 958R (5′-TCC GGC GTT GAM TCC AAT T-3′) (11). All PCR amplifications were performed by using the GeneAmp PCR kit with *Taq* DNA polymerase (Perkin Elmer, Inc.) in a PTC200 thermal cycler (MJ Research, Watertown, Mass.). Conditions for PCR were as follows: an initial denaturation step of 1.5 min at 94°C; 30 amplification cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), and elongation (30 s at 72°C); and a final extension step of 5 min at 72°C. The PCR products were purified by utilizing the QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, Calif.). Plasmid libraries for both domains were generated by ligating 2 μl of purified PCR fragments into the pCRII vector (Invitrogen, Carlsbad, Calif.) in accordance with the manufacturer's instructions. The ligation reactions were transformed into the *Escherichia coli* INVαF′ competent cells supplied with the Invitrogen Original TA Cloning Kit.

Library screening. Ninety-six randomly chosen clones were selected from colonies and grown overnight in Luria broth with kanamycin (100 μg/ml). The partial 16S rDNA fragments were amplified directly from 2 μl of an overnight-grown Luria broth culture added to 48 μl of PCR mixture using the following PCR conditions: 1 cycle of 3 min at 95°C; 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 5 min. Subsequently, the PCR products were digested separately with the restriction endonucleases *Hae*III and *Hha*I (New England Biolabs, Inc., Beverly, Mass.). The restriction digests were electrophoresed in a 3% Trevi-Gel (TreviGen, Gaithersburg, Md.) and visualized with SYBR Green I nucleic acid gel stain (Molecular Bio-Probes, Eugene, Oreg.) by using a Fluoroimager (Molecular Dynamics, Sunnyvale, Calif.). Clones were categorized according to their distinct RFLPs.

Sequencing and analysis. At least two representative clones for each unique RFLP were sequenced for comparative phylogenetic analysis. Plasmid DNA was purified with the Qiagen Plasmid Mini Kit (Qiagen, Inc.), and the sequence was determined after dye terminator cycle sequencing on an ABI 373 Automated Sequencer (Applied Biosystems, Foster City, Calif.). Initially, the clones were sequenced from the flanking 5′ end with a T7 sequencing primer and from the flanking 3′ end with an M13 reverse sequencing primer, both located on the pCRII vector, to obtain the complete fragment sequence.

Sequences were analyzed with the National Center for Biotechnology Information basic local alignment search tool via the BLASTN program (1) and the SIM_RANK program of the Ribosomal Database Project (28).

Chimeric sequence evaluation. Screening methods similar to those described previously by Snaidr et al. (29) were utilized for chimera screening. First, the sequences were manually aligned and then analyzed by using a software package that takes into account misalignments in secondary structure that could result from chimeras (7). Second, short sequences (~300 bp) of both the 16S rDNA 5′ and 3′ flanking regions were then submitted to both the BLASTN and SIM_RANK programs for comparative phylogenetic analysis of whole and partial gene sequences. Third, partial sequences were evaluated with the Check_Chimera program of the Ribosomal Database Project. To further minimize chimera formation, high-molecular-weight genomic DNA and PCR products were size fractionated in agarose gels prior to library construction. In addition, both bacterial and archaeal clone libraries were generated and screened from three replicate PCRs.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences used to generate a phylogenetic tree are as follows: *Clostridium litorale*, X77845; *Dehalobacter restrictus*, U84497; *Dehalococcoides ethenogenes*, AF004928; *Desulfitobacterium dehalogenans*, L28946; *Desulfitobacterium frappieri*, U40078; *Desulfobacter postgatei*, M26633; *Desulfomonile tiedjei*, M26635; *Desulfonema ishimotoei*, U45992; *Desulfosarcina variabilis*, M34407; *Desulfothiovibrio peptidovorans*, U52817; *Desulfotomaculum orientis*, M34417; *Desulfovibrio desulfuricans*, M34113; *Desulfuromonas acetexigens*, U23140; *Desulfuromusa succinoxidans*, X79415; *Fervidobacterium nodosum*, M59177; *Geobacter metallireducens*, L07834; *Geotoga petraea*, L10658; *Pelobacter propionicus*, X70954; *Petrotoga miotherma*, L10657; *Syntrophospora bryantii*, M26491; *Syntrophus gentianae*, X85132; *Thermoanaerobacter brockii*, L09165; *Thermosipho africanus*, M83140; *Thermotoga maritima*, M21774.

Sequences of the partial 16S rDNA clones exhibiting RFLP types 1, 4, 5, 11, 15, 17, 24, 25, 40, 105, 108, 109, and 144 were submitted to GenBank under accession no. AF058000 to AF058012, respectively.

RESULTS

Effects of PCB on community profiles. Selective enrichment techniques were used to establish *ortho*-dechlorinating enrichment cultures. Concomitantly, the cultures were monitored by screening the 16S rDNA community for putative PCB-*ortho*-dechlorinating microorganisms within these enrichment cultures. The diversity of the microbial community was minimized from the outset by the use of a minimal estuarine medium that contained sterilized Baltimore Harbor sediments. Further, the enrichment cultures were incubated with a single PCB congener, 2,3,5,6-CB, to facilitate monitoring of the rate and extent of dechlorination and to select for congener-specific dechlorinating organisms that were capable of reductively dechlorinating the parent congener and its trichlorinated intermediate (3).

Enrichment cultures that exhibited *ortho* dechlorination of 2,3,5,6-CB were generated by three sequential transfers (10% inoculum) of Baltimore Harbor sediments in estuarine medium supplemented with a mixture of three fatty acids: propionate, butyrate, and acetate (3, 9). Following the third sequential transfer, the only dechlorination pathway observed for these cultures, *ortho* dechlorination of 2,3,5,6-CB (Fig. 1A,

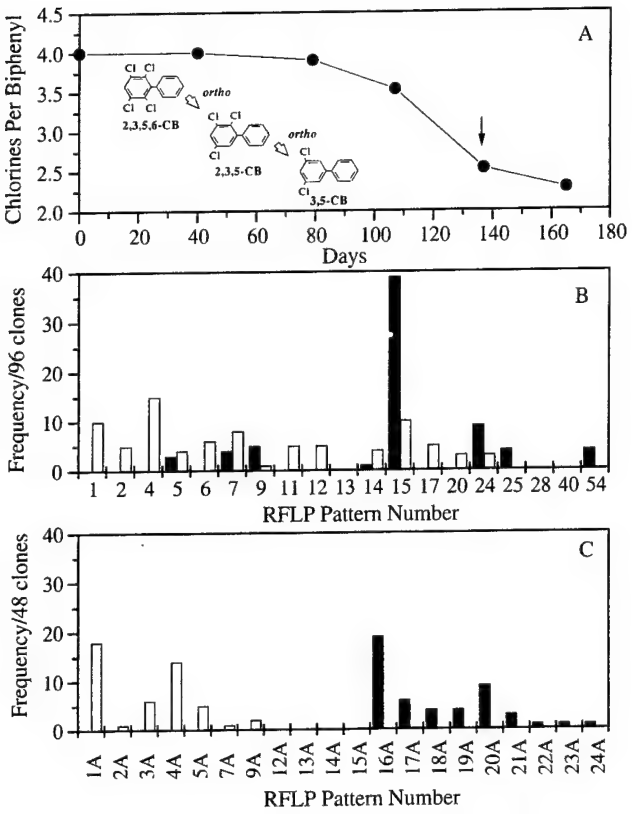

FIG. 1. (A) Rate of chlorine removal from 2,3,5,6-CB by enrichment cultures containing 0.1% Baltimore Harbor sediment. The dechlorination pathway of 2,3,5,6-CB by *ortho*-dechlorinating enrichment cultures is shown in the inset. (B) Community profiles of bacterial 16S rDNA clones from Baltimore Harbor enrichment cultures incubated with (□) and without (■) 2,3,5,6-CB. Samples for phylogenetic analysis were taken at day 137, as indicated for panel A. Both enrichment cultures were amended with a mixture of three fatty acids as carbon sources. (C) Community profiles of archaeal 16S rDNA clones from Baltimore Harbor enrichment cultures incubated with (□) and without (■) 2,3,5,6-CB.

inset), was observed in the PCB-containing culture after 79 days and achieved a maximum rate after 107 days (Fig. 1A). Approximately 75% of the parent congener was converted to 3,5-CB after 160 days. Duplicate enrichment cultures that did not contain a PCB were maintained and sequentially transferred concurrently with the PCB-dechlorinating enrichment cultures. Both dechlorinating and nondechlorinating enrichment cultures were methanogenic.

Community profiles analyzed at 137 days after the third sequential transfer of dechlorinating and nondechlorinating enrichment cultures are shown in Fig. 1B. Sixteen predominant RFLP types were identified in the cultures, and 16S rDNA fragments from two representative clones for each pattern were subjected to comparative sequence analysis. Eight RFLP types, 1, 2, 4, 6, 11, 12, 17, and 20, were detected exclusively in cultures that contained the PCB congeners. RFLP type 4, the most predominant clone, accounting for 30% of the selected clones, showed the highest sequence similarity to the δ subgroup (Table 1). RFLP type 1, the second most predominant clone, accounted for 20% of the selected clones and showed the highest sequence similarity to the *Thermotogales* subgroup. Of the remaining clones, RFLP types 11 and 12 had the highest sequence similarity to the low-G+C gram-positive subgroup, RFLP types 4, 6, and 20 had the highest sequence homology to members of the δ subgroup, and RFLP type 17 exhibited the highest sequence similarity to the deeply branching species *Dehalococcoides ethenogenes* (25). Only one representative clone with RFLP type 6 was identified because the partial 16S rDNA insert was unstable and often lost from the vector prior to sequencing.

RFLP types 7 and 14 showed the highest sequence similarity to the low-G+C gram-positive subgroup. Both patterns were detected in the presence and absence of a PCB but increased significantly (≥50%) in medium that contained a PCB. The remaining clones, which had high sequence similarity to members of the δ subgroup (RFLP type 25) and the low-G+C gram-positive subgroup (RFLP types 5, 9, 15, 24, and 54), were either detected at similar frequencies in both cultures, increased in the frequency of detection relative to one another, or detected only in the PCB-free culture. The results suggest that species represented by the latter clones do not have a significant role in PCB *ortho* dechlorination.

The community profiles of methanogenic archaea enriched in the presence and absence of a PCB differed significantly (Fig. 1C). Seven predominant RFLP types were detected in the actively dechlorinating culture. RFLP types 1A, 4A, and 5A had the highest sequence similarity to the *Methanosarcinales* subgroup, whereas RFLP types 2A, 3A, 7A, and 9A had the highest sequence similarity to the *Methanomicrobiales* subgroup (Table 2). Conversely, none of the clones detected in the presence of a PCB were detected in the PCB-free enrichment culture. RFLP types 16A, 19A, 20A, 21A, 22A, and 24A had the highest sequence similarity to the *Methanosarcinales* subgroup, and the remaining clones, with RFLP types 17A, 18A, and 23A, had the highest similarity to the *Methanomicrobiales* subgroup. Although the community profiles differed in the absence and presence of a PCB congener, both cultures exhibited similar distributions of species belonging to the autotrophic, hydrogen-utilizing order *Methanomicrobiales* and the aceticlastic and methylotrophic order *Methanosarcinales*. This preliminary

TABLE 1. Phylogenetic affiliations of predominant RFLP types from PCB-*ortho*-dechlorinating enrichment cultures based on bacterial 16S rRNA gene sequences

RFLP type	Closest phylogenetic relative	% Similarity to closest relative
1	*Thermotoga maritima*	85
2	*Bacteroides eggerthii*	89
4	*Desulfosarcina variabilis*	93
5	*Desulfothiovibrio peptidovorans*	87
6	*Desulfuromonas thiophila*	94
7	*Clostridium litorale*	91
9	*Desulfonema magnum*	82
11	*Syntrophospora bryantii*	94
12	Unidentified oil field bacterium	75
15	*Clostridium litorale*	99
17	*Dehalococcoides ethenogenes*	89
20	*Pelobacter acidigallici*	86
24	*Acholeplasma laidlawii*	84
25	*Desulfonema magnum*	94
28	*Desulfovibrio caledoniensis*	95
40	*Syntrophus gentianae*	94
54	*Clostridium litorale*	84
105	*Desulfuromonas thiophila*	96
108	*Desulfuromonas acetexigens*	99
109	*Desulfovibrio* sp.	92
116	*Desulfovibrio* sp.	86
130	Uncultured eubacterium	89
138	Unidentified low-G+C gram-positive sp.	96
144	*Desulfovibrio* sp. strain B650	98
146	*Desulfovibrio* sp.	91

TABLE 2. Phylogenetic affiliations of predominant RFLP types from PCB-*ortho*-dechlorinating enrichment cultures based on archaeal 16S rRNA gene sequences

RFLP type	Closest phylogenetic relative	% Similarity to closest relative
1A	*Methanosaeta concilii*	91
2A	*Methanoculleus marisnigri*	90
3A	*Methanoplanus limicola*	90
4A	*Methanohalophilus mahii*	87
5A	*Methanohalobium evestigatum*	81
7A	*Methanogenium organophilum*	96
9A	*Methanospirillum hungatei*	87
16A	*Methanosaeta concilii*	99
17A	*Methanoplanus petrolearius*	94
18A	*Methanogenium organophilim*	96
19A	*Methanosaeta concilii*	96
20A	*Methanohalophilus mahii*	86
21A	*Methanosaeta concilii*	96
22A	*Methanosaeta concilii*	99
23A	*Methanoplanus limicola*	92
24A	*Methanosaeta concilii*	99

characterization represented a baseline community profile for the PCB-dechlorinating and nondechlorinating enrichment cultures.

Effects of Baltimore Harbor sediment on *ortho*-dechlorinating consortia. To eliminate the effects of putative alternative electron acceptors (e.g., humic acids, SO_4^{2-}, Fe^{2+}) and undefined nutrients that may be present in Baltimore Harbor sediments, PCB-dechlorinating enrichment cultures were sequentially transferred in completely defined estuarine medium that contained 2,3,5,6-CB and three fatty acids as carbon sources without the addition of sterile sediments (9). After four sequential transfers in the absence of sediments, dechlorination of 2,3,5,6-CB was detected after an extensive lag period (>100 days) and the congener was completely transformed to 3,5-CB after 240 days (Fig. 2A). Methane production was observed in the sediment-free enrichment cultures.

Community profiles were compared before and after the onset of dechlorination in the fourth sequential enrichment culture transfer in defined medium (Fig. 2B). Of the 14 predominant RFLP types previously detected in PCB-dechlorinating cultures with sediment, 10 were detected in the sediment-free cultures. As observed in the previous cultures, RFLP type 1 was the predominant species, accounting for 36% of the clones detected. Of the seven remaining RFLP types that appeared exclusively in the PCB-dechlorinating enrichment culture with sediment, only four were detected in the absence of sediment (RFLP types 4, 6, 11, and 17) and only the relative detection frequencies of RFLP type 5 increased significantly with the onset of dechlorination. The absence of RFLP types 2, 9, 12, 14, 20, and 54 indicated that these species were diluted out to undetectable levels after sediment was deleted. Although this observation suggests that the latter species are not required for *ortho* dechlorination of 2,3,5,6-CB, it does not rule out the possibility that they are capable of dechlorination but lacked specific growth factors provided by the sediments. The three remaining clones, RFLP types 28, 40 (δ subgroup), and 13 (low-G+C gram-positive subgroup), were not observed previously in medium that contained sediment but were selectively enriched in the absence of sediment.

Overall, the most predominant members of the methanogenic archaeal community did not change significantly with the onset of dechlorination in the sediment-free enrichment cultures, as indicated in Fig. 2C, and all were observed in previous cultures with sediment and the PCB congener. RFLP types 4A, 12A, and 14A were detected only after dechlorination was observed in the enrichment. RFLP types 3A, 5A, and 13A were detected both in the preactive and active cultures. RFLP type 15A was detected only in the absence of dechlorination. RFLP type 5A, the most predominant clone, had the highest sequence homology to members of the order *Methanosarcinales*, whereas the second most predominant clone, RFLP 3A, had the highest homology to members of the order *Methanomicrobiales*.

Effects of carbon source on *ortho*-dechlorinating consortia. PCB-dechlorinating enrichment cultures grown with three fatty acids were sequentially transferred into defined estuarine medium that contained 2,3,5,6-CB and sediment with sodium acetate as the sole electron donor to minimize community diversity further. After three sequential transfers, dechlorination was detected within 28 days and the congener was completely transformed to 3,5-CB after 85 days (Fig. 3). Growth rates were not measured in cultures that contained sediment due to turbidity caused by the particles. However, enrichment cultures that contained sodium acetate had higher dechlorination rates than cultures that contained a mixture of three fatty acids. Cultures were methanogenic with sodium acetate.

Community profiles were determined after three sequential transfers of the enrichment cultures with 2,3,5,6-CB and so-

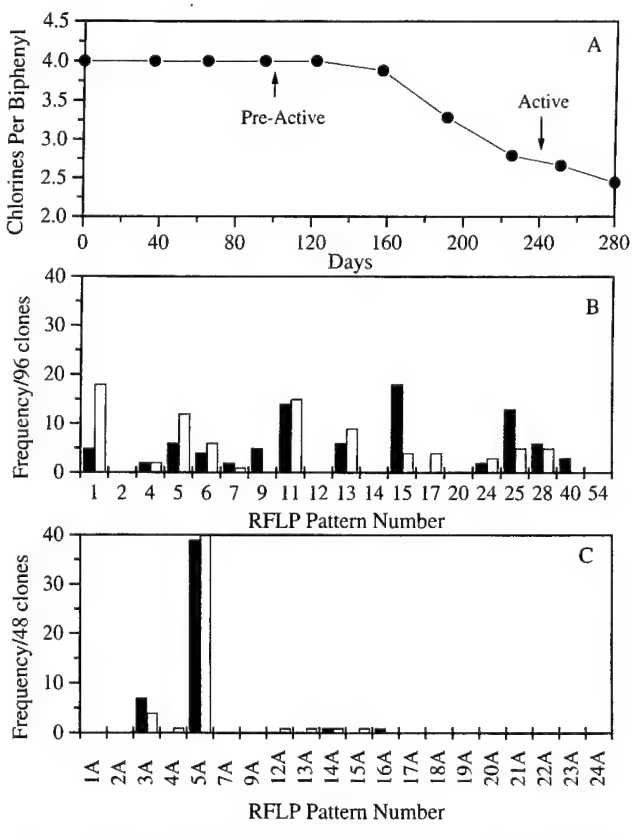

FIG. 2. (A) Reductive dechlorination of 2,3,5,6-CB in sediment-free Baltimore Harbor enrichment cultures with a mixture of three fatty acids as carbon sources. Sediment was removed by dilution after four sequential transfers. The enrichment culture was sampled for phylogenetic analysis prior to the onset of dechlorination (preactive, day 102) and during *ortho* dechlorination (active, day 240). (B) Community profiles of bacterial 16S rDNA clones from sediment-free Baltimore Harbor enrichment cultures prior to (■) and following (□) the onset of *ortho* dechlorination. (C) Community profiles of archaeal 16S rDNA clones from sediment-free Baltimore Harbor enrichment cultures prior to (■) and following (□) the onset of *ortho* dechlorination.

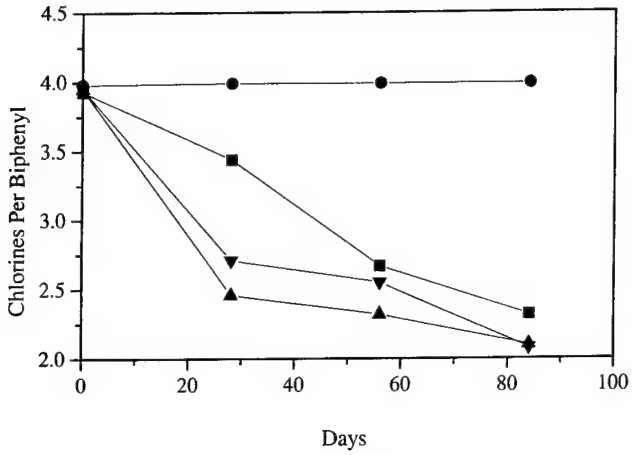

FIG. 3. Dechlorination rates of Baltimore Harbor cultures treated with physiological inhibitors. Symbols: ▲, no inhibitor; ■, 3 mM BES; ●, 20 mM sodium molybdate; ▼, 100-μg/ml vancomycin.

dium acetate (Fig. 4A). Only 6 RFLP types, 6, 7, 15, 17, 24, and 40, of the 19 predominant RFLP types detected in the previous cultures that contained fatty acids were detected in cultures that contained only acetate as an electron donor. Interestingly, RFLP types 4, 5, 11, 13, 25, and 28, which were predominant in cultures that contained a mixture of fatty acids that included sodium acetate, were not detected in dechlorinating enrichment cultures grown with sodium acetate alone. These results suggest that growth of the latter species was linked to butyrate or propionate catabolism. The shift to acetate resulted in a significant overall change in the community. The most predominant RFLP types (105, 108, 109, and 116; frequency, ≥2/96 clones) detected in enrichment cultures containing sodium acetate were not detected previously, indicating that their growth may be linked specifically to acetate. All of the predominant RFLP types belonged to the δ subgroup.

Effects of selective inhibitors on *ortho*-dechlorinating consortia. To further reduce community diversity and select for microbial species linked to *ortho* dechlorination of 2,3,5,6-CB with sodium acetate as the growth substrate, enrichment cultures were transferred into medium that contained physiological inhibitors. The inhibitors included BES, which selectively inhibits the methanogenic archaea (16); sodium molybdate, an analogue of sulfate, which selectively inhibits sulfate-reducing bacteria (31); and vancomycin, which selectively inhibits gram-positive bacteria by inhibiting biosynthesis of the cell wall peptidoglycan (27). Active cultures were transferred to medium that contained the selected physiological inhibitor and then sampled for analysis of the 16S rDNA community profile after the onset of dechlorination.

The addition of BES only slightly inhibited the rate of dechlorination, and nearly complete dechlorination of 2,3,5,6-CB to 3,5-CB occurred within 85 days (Fig. 3). The bacterial diversity and relative numbers of bacterial species in the BES-treated culture closely resembled those in untreated control cultures (Fig. 4A and B). Seven previously undescribed RFLP types were detected, but only RFLP type 130 (low-G+C gram-positive subgroup) was predominant at frequencies of ≥2/96 clones sampled. However, methanogenesis did not occur and archaeal rDNA was not detected by PCR, indicating that the methanogenic archaea were not required for *ortho* dechlorination of 2,3,5,6-CB to 2,3,5-CB and 3,5-CB with sodium acetate.

As expected, vancomycin caused a more significant shift in the bacterial community than BES (Fig. 4C). Interestingly, vancomycin, like BES, also inhibited methanogenesis and precluded detection of archaeal rDNA by PCR, confirming that the methanogenic archaea were not required for *ortho* dechlorination of 2,3,5,6-CB with sodium acetate. Five RFLP types, 6, 7, 17, 24, and 105, were detected previously in PCB-dechlorinating cultures that did not contain an inhibitor. Of the 10 RFLP types not detected previously, the two most predominant (frequency, ≥2/96 clones), 144 and 146, were most closely related to the δ subgroup.

The addition of sodium molybdate (final concentrations of 2 and 20 mM) completely inhibited dechlorination and inhibited methanogenesis of 2,3,5,6-CB (Fig. 3). Furthermore, the genomic yield of this culture was approximately 10-fold lower than that of the previous cultures, and the bacterial diversity was significantly reduced (Fig. 4D). As expected, RFLP types 40, 105, 108, 109, and 116, which had sequence similarity to the δ subgroup, were not detected in the molybdate culture. However, the relative detection frequency of RFLP type 6, which is also phylogenetically related to the δ subgroup, was similar to that of the positive control, along with low-G+C gram-positive RFLP types 7, 15, and 24. RFLP type 138 (low-G+C gram-

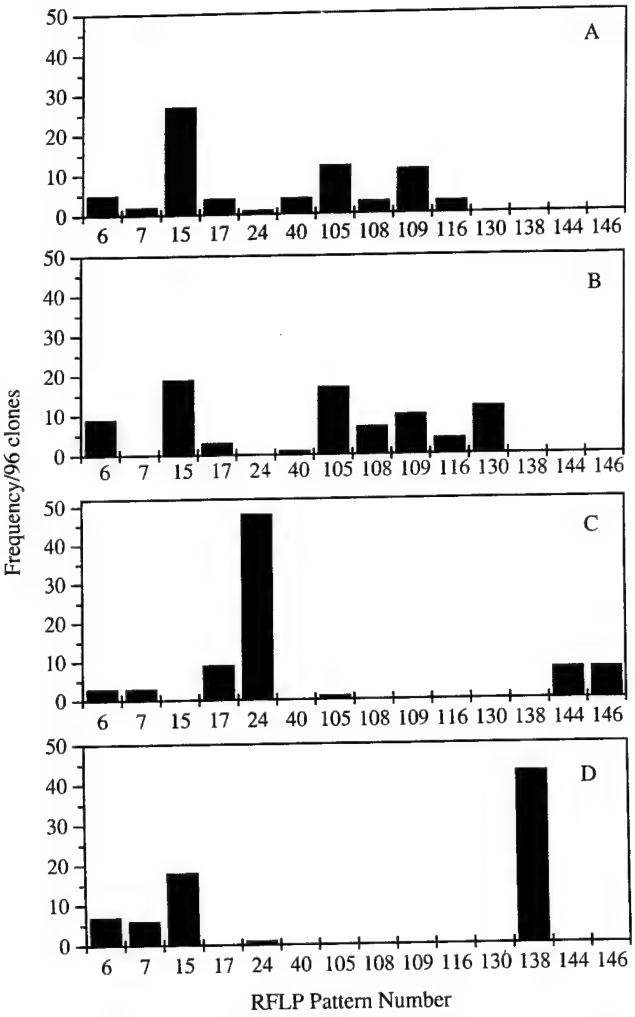

FIG. 4. Effects of physiological inhibitors on community profiles of Baltimore Harbor enrichment cultures enriched with 2,3,5,6-CB, acetate, and 0.1% Baltimore Harbor sediment. Panels: A, no inhibitor; B, 3 mM BES; C, 100-μg/ml vancomycin; D, 20 mM sodium molybdate.

positive subgroup) was detected only in this culture and, therefore, was unlikely to represent an *ortho*-dechlorinating species.

DISCUSSION

Molecular screening of the 16S rDNAs from the total community of genomic DNAs was used to characterize microbial consortia in PCB-*ortho*-dechlorinating enrichment cultures without isolation of heretofore unculturable dechlorinating species. Bias can be introduced at various stages in the protocol, particularly during cell lysis and PCR amplification. Therefore, to minimize screening bias, a physical cell lysis method, bead mill homogenization, was used to effectively lyse all cell types, including those most recalcitrant to physical and enzymatic treatments (22, 26). To minimize PCR bias, separate primers were used for bacterial and archaeal phylogenetic domains. The primers were tested with Baltimore Harbor enrichment cultures and determined empirically to yield greater community diversity than other "universal" primers previously described (data not shown). In addition, PCR parameters, including use of a denaturant (formamide), temperature, and ion concentration, were optimized to yield maximum diversity in the community profiles of Baltimore Harbor enrichment cultures. Other factors, such as species-specific 16S rDNA copy number and PCR bias for a low-G+C template, also affect the quantitative assessment of microbial communities (14), and as a result, this approach can provide only an estimate of the actual abundance of microorganisms in each enrichment. In the current study, all enrichment cultures were sequentially transferred from the same inoculum source and grown under similar conditions. Throughout the study, community profile comparisons of duplicate cultures and of sequential transfers of identical treatments were reproducible (data not shown). Therefore, it was possible to determine whether an individual species was associated with PCB dechlorination by assaying for the coexistence or mutual exclusion of its RFLP type with dechlorination after treatment with physiological inhibitors. By monitoring the rates of dechlorination and relative frequencies of detection of specific RFLP types associated with PCB dechlorination, this approach was used to establish a highly defined PCB-*ortho*-dechlorinating community and to monitor the effects of sequential culture transfers and treatments on specific community members.

Previous attempts to identify and isolate anaerobic PCB dechlorinators by selective enrichment and isolation techniques have been unsuccessful (2). The failure to identify these species is likely due to the development of previous enrichment cultures in complex, undefined medium, which resulted in selection for faster-growing, non-PCB-dechlorinating microorganisms that likely outcompete PCB dechlorinators. By using the SEMM approach, conditions were developed that would maintain cultures of PCB-dechlorinating consortia indefinitely in a defined minimal medium. While other molecular approaches have been described for the isolation of bacteria from the environment (19, 23, 33), this is the first reported application of a molecular approach for the development of a defined PCB-dechlorinating consortium in a minimal medium. By reducing the medium complexity, the community diversity in a PCB-dechlorinating consortium was systematically reduced with the addition of medium components and physiological inhibitors that selectively promoted the growth of species involved in *ortho* dechlorination of 2,3,5,6-CB. Screening of the microbial communities by RFLP of PCR-amplified 16S rDNA as the cultures were selectively enriched provided a means for effectively monitoring the effects of treatments on individual species and, by a process of elimination, enabled us to identify species that are most likely to catalyze PCB dechlorination. In addition, the phylogeny of individual RFLP types was determined by comparative sequence analysis of the PCR-amplified 16S rDNA fragments (Fig. 5).

By sequentially transferring cultures in both the presence and the absence of 2,3,5,6-CB, species that had a selective growth advantage with the congener were enriched, as indicated by differences in the community profiles. However, several RFLP types were present under both culture conditions, indicating that these species utilized alternative electron acceptors to PCB for growth. Possible mechanisms included (i) methanogenic carbon dioxide reduction by hydrogen-utilizing methanogens via interspecies hydrogen exchange with propionate- and butyrate-utilizing acetogens or acetate-dismutating species, which include low-G+C gram-positive species such as clostridia and members of the δ subgroup; (ii) dismutation of acetate by aceticlastic methanogens; (iii) fatty acid oxidation with unknown dissimilatory electron acceptors in sediment; and (iv) fatty acid oxidation with PCB as a dissimilatory electron acceptor.

To further reduce selection to growth-linked or cometabolic PCB dechlorination, enrichment cultures were initiated and sequentially transferred into totally defined sediment-free medium. Although the medium complexity was reduced, the overall community diversity was reduced only slightly and the same phylogenetic groups (the δ, low-G+C gram-positive, *Thermotogales*, and *Dehalococcoides* subgroups) were detected, indicating that most species from the initial enrichment cultures adapted to growth without sediments. Past reports have indicated that sediments were required in order to maintain microbially mediated PCB-dechlorinating activity through sequential transfers, and several possible roles for sediment in the dechlorination process are discussed by Cutter et al. (9) and Boyle et al. (5). By developing a microbial community adapted to growth in defined medium, it was possible to further reduce the complexity of the *ortho*-dechlorinating community systematically by eliminating or substituting components.

The influence of the carbon source on the community of PCB-dechlorinating enrichment cultures was investigated. Changing the carbon source from a mixture of butyrate, propionate, and acetate to acetate as the sole electron donor caused a dramatic shift in the microbial community. Although the growth rates observed in enrichment cultures with the mixture of fatty acids were greater than rates observed in cultures with acetate alone, the dechlorination rate was greater in enrichment cultures that contained acetate alone. It is well documented that enrichment conditions, choice of PCB congener, and source of inoculum can influence dechlorinating activities (2). However, this is the first confirmed report of the influence of an electron donor on the community profile of a PCB-dechlorinating enrichment culture.

The overall results of this study show that the defined growth conditions supported the growth of only four phylogenetic subgroups among the bacteria, i.e., the δ, low-G+C gram-positive, and *Thermotogales* subgroups and a single species near the deeply branching species *D. ethenogenes*, and two phylogenetic subgroups among the archaea, i.e., the H_2-CO_2 utilizing *Methanomicrobiales* subgroup and the methylotrophic and aceticlastic *Methanosarcinales* subgroup (Fig. 5). The detection of the H_2-CO_2-utilizing methanogens indicates that hydrogen was likely generated by fatty acid-oxidizing acetogenic bacteria. This conclusion is supported by the observation that H_2-CO_2-utilizing *Methanomicrobiales* and methylotrophic and aceticlastic *Methanosarcinales* subgroup species are evenly distributed when enrichment cultures are grown on a mixture of fatty

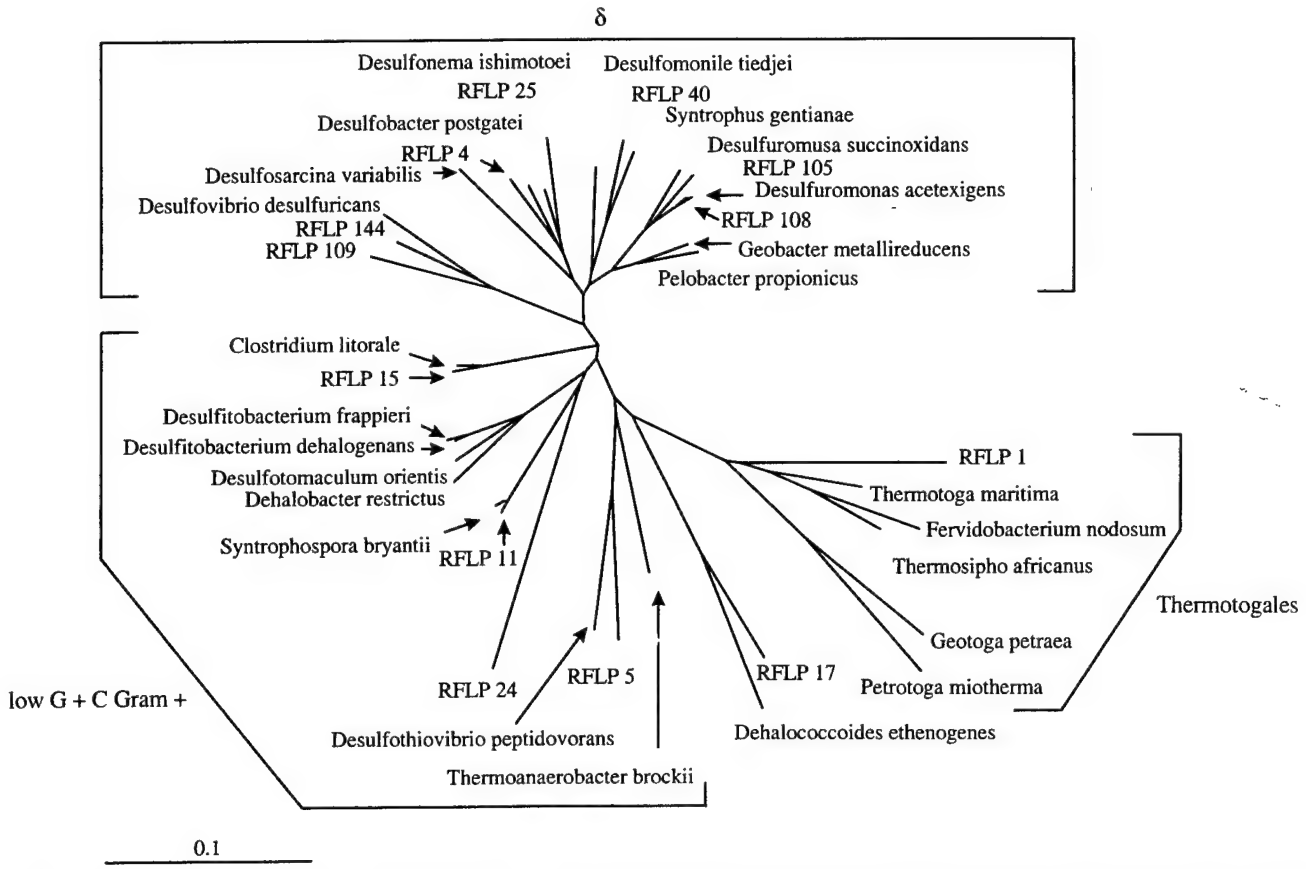

FIG. 5. Phylogenetic tree inferred from comparative sequence analyses of partial 16S rDNA sequences from several predominant clones obtained from PCB-*ortho*-dechlorinating enrichment cultures. For construction of a phylogenetic tree, approximately 890-bp segments of selected sequences were aligned manually with a collection of known bacterial 16S rRDAs (for nucleotide sequence accession numbers, see Materials and Methods) obtained from the GenBank database by using software described by Chun (7). Evolutionary distances, expressed as estimated changes per 100 nucleotides, were calculated from the percentages of similarity by using the correction of Jukes and Cantor (18). A dendogram was constructed with PHYLIP based on the unweighted pair group method with arithmetic averages (15). The bar represents 0.1 U of evolutionary distance.

acids, but *Methanosarcinales* species become most predominant with acetate only. However, dechlorination was observed when methanogenesis and growth of all methanogenic archaea were inhibited by BES, indicating that methanogenic archaea are not required for acetate-mediated *ortho* dechlorination of 2,3,5,6-CB. The slight inhibition of dechlorination with BES treatment likely resulted from nonspecific inhibition of bacterial species that were involved in dechlorination. This conclusion is further supported by the observation that vancomycin treatment also inhibited methanogenesis and methanogen growth but had only a slight effect on the rate of dechlorination. A report by May et al. indicated that colonies of PCB-enriched consortia plated on solidified media *para* and/or *meta* dechlorinated 2,3,4-CB and 2,4,5-CB in the absence of methanogenesis (24). In contrast, the same cultures lost the ability to dechlorinate 2,5,3′,4′-CB and 3,4,2′-CB concurrently with the loss of methanogenic activity. Likewise, Ye et al. (38) reported that methanogenesis occurred concurrently with process H (*meta*, *para*) dechlorination of Aroclor 1242 but that process M (*meta*) dechlorination occurred in the absence of methanogenesis. Results of the current study show that *ortho* dechlorination of 2,3,5,6-CB is catalyzed in the absence of methanogenesis. These results, in conjunction with previous reports on *para* and *meta* dechlorination of individual congeners and Aroclors, support the hypothesis that different phylogenetic groups of bacteria and archaea dechlorinate selected PCB congeners.

RFLP type 15, which had high sequence similarity to *Clostridium* sp., was inhibited by the addition of vancomycin but not by molybdate. Reduction in the relative abundance of RFLP type 15 by the addition of vancomycin or by the removal of sediment did not affect the rate of removal of *ortho* chlorines from 2,3,5,6-CB, which suggests that RFLP type 15 does not have a role in dechlorination. Following pasteurization (80°C for 1 h) of cultures containing fatty acids and sediment, *ortho* dechlorination ceased, further supporting the conclusion that spore-forming microbes such as *Clostridium* spp. are not responsible for *ortho* dechlorination. In contrast, *para* and *meta* dechlorination of Aroclor 1242 by Hudson River sediments was shown to be resistant to pasteurization (36). Davenport et al. have reported that archaeal and clostridial 16S sequences are predominant in microcosms that *meta* and *para* dechlorinate 2′,3,4-CB (10). However, neither of the latter two studies reported *ortho* dechlorination, which further supports the hypothesis that different species exhibit congener specificity.

Species most frequently associated with *ortho* dechlorination of 2,3,5,6-CB in the Baltimore Harbor enrichment cultures had high sequence similarities to described species of dissimilatory sulfur- and sulfate/iron-reducing bacteria. In the presence of molybdate, *ortho* dechlorination of 2,3,5,6-CB was inhibited. Further, with the exception of one species, all of the 16S rDNA clones frequently associated with actively dechlorinating cultures cluster with the sulfate/iron-dissimilating δ subgroup or

the elemental sulfur/thiosulfate/sulfite-dissimilating low-G+C gram-positive and *Thermotogales* subgroups. Ye proposed that spore-forming dissimilatory sulfate-reducing bacteria were responsible for process M (*meta*) dechlorination, since pasteurization and ethanol treatment did not inhibit dechlorinating activity in freshwater cultures but addition of molybdate did inhibit activity (39). In addition, described species that reductively dechlorinate aromatic or aliphatic compounds also cluster with sulfate or sulfur/iron reducers in the δ subgroup (e.g., *Desulfomonile tiedjei*, *Pelobacter* sp. TT4B strain 2CP1) and with the sulfur/thiosulfate/sulfite reducers in the low-G+C gram-positive subgroup (e.g., *Desulfitobacterium dehalogenans* and *Desulfitobacterium frappieri*) (4, 8, 12, 20, 35). Although species related to the *Thermotogales* subgroup have not been previously implicated in reductive dechlorination, several members of this phylum are capable of S^0 reduction. Another species that was detected in *ortho*-dechlorinating enrichment cultures had the highest sequence similarity to the deeply branching species *Dehalococcoides ethenogenes*, which has been described as an obligate perchloroethylene-dechlorinating species (25). The consistent detection of this species in actively PCB-*ortho*-dechlorinating cultures and its absence from nondechlorinating cultures present the intriguing possibility that other obligate dehalogenating species exist.

In summary, SEMM has been shown to be an effective approach for developing community profiles associated with specific PCB-dechlorinating activities in a minimal defined medium. By using this approach, we have demonstrated that highly defined *ortho*-dechlorinating enrichment cultures have been developed and a stable microbial community has been maintained throughout sequential transfers in minimal growth conditions. Based on nutrient requirements of known species closely related to species identified in these *ortho*-dechlorinating enrichment cultures, efforts are currently under way to isolate and further characterize species from the enrichment community to confirm their role in catalysis of the dechlorination process.

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Microbial Reductive Dechlorination of Aroclor 1260 in Anaerobic Slurries of Estuarine Sediments

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Reductive dechlorination of Aroclor 1260 was investigated in anaerobic slurries of estuarine sediments from Baltimore Harbor (Baltimore, Md.). The sediment slurries were amended with 800 ppm Aroclor 1260 with and without the addition of 350 μM 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-CB) or 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB) and incubated in triplicate at 30°C under methanogenic conditions in an artificial estuarine medium. After 6 months, extensive *meta* dechlorination and moderate *ortho* dechlorination of Aroclor 1260 occurred in all incubated cultures except for sterilized controls. Overall, total chlorines per biphenyl decreased by up to 34%. *meta* chlorines per biphenyl decreased by 65, 55, and 45% and *ortho* chlorines declined by 18, 12, and 9%, respectively, when 2,3,4,5-CB, 2,3,5,6-CB, or no additional congener was supplied. This is the first confirmed report of microbial *ortho* dechlorination of a commercial polychlorinated biphenyl mixture. In addition, compared with incubated cultures supplied with Aroclor 1260 alone, the dechlorination of Aroclor 1260 plus 2,3,4,5-CB or 2,3,5,6-CB occurred with shorter lag times (31 to 60 days versus 90 days) and was more extensive, indicating that the addition of a single congener stimulated the dechlorination of Aroclor 1260.

Polychlorinated biphenyls (PCBs) and other anthropogenic pollutants adsorb to sediments due to the hydrophobic nature of the compounds. As sediments settle, adsorbed PCBs accumulate in the lower anoxic layers of the sediment column, where reductive dechlorination of PCBs by anaerobic microorganisms has been demonstrated to occur in the laboratory and in situ (1, 2, 4, 5, 10, 12, 22). The turnover of naturally formed halogenated organics in marine coastal regions suggests that these environments have a significant potential for dechlorination (14, 18). However, few studies have focused on the dechlorination of PCBs in marine and estuarine sediments (2, 11, 20).

Anaerobic PCB dechlorination has the potential to reduce the toxicity of the PCBs (5, 11, 23) and convert highly persistent congeners, frequently the more extensively chlorinated congeners, into forms that are more amenable to aerobic degradation (6, 8, 13, 15, 26). However, only the loss of *meta* and/or *para* chlorines has been demonstrated when preexisting or freshly added commercial PCB mixtures (e.g., Aroclors 1242, 1254, and 1260, etc.) have been microbially dechlorinated in sediments from the Hudson River (N.Y.), Silver Lake (Pittsfield, Mass.), Woods Pond (Lenox, Mass.), and Puget Sound (2, 3, 7, 20, 21). *ortho* dechlorination of an Aroclor has not been demonstrated, and microbial dechlorination of Aroclor 1260, preexisting or freshly added to sediments, has not been very extensive. The addition of single PCB congeners, in a process called priming, stimulated the dechlorination of Aroclor 1260 residue in Woods Pond sediments, but priming did not promote *ortho* dechlorination of the residual Aroclor 1260 (3, 5, 7, 31).

Baltimore Harbor (BH; Baltimore, Md.) has been heavily impacted by industrial activity over the last 150 years, and PCBs have accumulated in sediments throughout the harbor (27). We recently reported that the single congeners 2,3,5,6-chlorobiphenyl (2,3,5,6-CB), 2,3,5-CB, and 2,3,6-CB were *ortho* dechlorinated by enrichment cultures that contained sediments collected from the northwest branch of the harbor (9). Here we describe the anaerobic dechlorination of Aroclor 1260 by enrichment cultures prepared with these sediments. The data demonstrate extensive *meta* dechlorination and moderate *ortho* dechlorination. Furthermore, we show that the *meta* and *ortho* dechlorinations are stimulated by the addition of single PCB congeners.

MATERIALS AND METHODS

Sediment collection and storage. Collection of estuarine sediments from BH was described previously (9), and the sediment samples were stored anaerobically at room temperature for 14 months in the dark before use in these experiments. No background PCBs were detected in these sediments based on methods described below (detection limit, ~0.01 μg/g of PCB standard used).

Preparation of slurries and incubation. Estuarine medium without sulfate (E-Cl) was prepared as described by Berkaw et al. (9). In an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) containing 95% nitrogen–5% hydrogen, sediment slurries were prepared by mixing 1 volume of wet BH sediment with 4 volumes of E-Cl medium (equivalent to 0.06 g [dry weight] of sediment per ml). Aliquots of the slurries (30 ml) were dispensed into 50-ml serum bottles and allowed to stand for 5 days in the anaerobic chamber.

To prepare sterile controls, slurries were autoclaved twice for 1 h at 121°C on 2 consecutive days. Live cultures and sterile controls prepared in triplicate were amended with 800 ppm Aroclor 1260 (800 μg per g [dry weight] of sediment or 133 μmol per liter of slurry) and either 350 μM (μmol per liter of slurry) 2,3,4,5-CB, 350 μM 2,3,5,6-CB, or no additional congener. All enrichment cultures were incubated at 30°C in the dark. Each month, all enrichments were supplemented with a fatty acid mixture (2.5 mM each acetate, propionate, and butyrate).

Sample preparation, extraction, and analysis. The dechlorination of 2,3,4,5-CB, 2,3,5,6-CB, and Aroclor 1260 in each culture was analyzed at various time points throughout a 6-month period. Samples were drawn and extracted in ethyl acetate (high-performance liquid chromatography grade; Fisher Scientific, Pittsburgh, Pa.), and the organic fraction was passed over a Florisil-copper column as described previously (9). PCBs were analyzed with a Hewlett-Packard 5890 series II gas chromatograph (GC) equipped with an RTX-1 capillary column (30 m by 0.25 mm [inside diameter] by 0.25 μm; Restek Corp., Bellefonte, Pa.) and a Ni^{63} electron capture detector as described previously (9).

Congeners 2,3,4,5-CB and 2,3,5,6-CB and their dechlorination products were identified by matching their retention times with those of authentic standards (>90% purity; AccuStandard, New Haven, Conn.) and were quantified by use of

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a piecewise-fit calibration curve generated from these standards at 9 to 16 calibration levels (9). PCB congeners in Aroclor 1260 and their dechlorination products were identified by matching their GC retention times with a customized PCB standard prepared by supplementing Aroclor 1260 with the dechlorination products observed in Woods Pond (24) or a standard mixture composed of 3-3-CB, 3-4-CB, 3,5-3-CB, 3,5-4-CB, 2,4-3,5-CB, and 2,5-3,5-CB. Congener assignments were made in accordance with those reported by Frame et al. (16). Each congener in the Aroclor mixture was quantified by use of a piecewise-fit calibration curve generated from standards at 4- to 8-point calibration levels. Congener and homolog distributions for each sample were calculated and reported in units of moles percent. Congener distributions for each enrichment culture with Aroclor and 2,3,4,5-CB (or 2,3,5,6-CB) were calculated after subtracting the peaks corresponding to 2,3,4,5-CB (or 2,3,5,6-CB) and their potential dechlorination products. Therefore, values for dechlorination of Aroclor 1260 in those incubations are conservative.

Mass selective analysis was performed with a Hewlett-Packard 6890 series GC equipped with an HP-5MS capillary column (30 m by 0.25 mm [inside diameter] by 0.25 μm; Hewlett-Packard, Atlanta, Ga.) and a Hewlett-Packard 6890 series mass selective detector (MS). Chromatographic conditions were identical to those described previously for the GC-electron capture detector (9). Our analysis found that 2,4-3,5-CB was not resolved with 2,3,6-2,6-CB on a DB-1 column. Thus, we used GC-MS to identify 2,4-3,5-CB (*m/z* 292) and 2,3,6-2,6-CB (*m/z* 326) due to different molecular formulas between these two congeners. In addition, analysis of our PCB standard mixtures on an HP-5MS capillary column resulted in the resolution of 2,4-3,5-CB from 2,3,6-2,6-CB. We also found that 2,5-3,5-CB was not resolved from 2,3,4-2-CB, 2,3,6-4-CB, and 2,6-3,4-CB on a DB-1 column as reported previously (16). However, 2,5-3,5-CB was resolved from these congeners by using an HP-5MS column with GC-MS.

RESULTS

Dechlorination of Aroclor 1260 in BH enrichment cultures was detected within 4 months (Fig. 1). However, the lag time decreased to 31 days in sediment slurries additionally supplied with 2,3,4,5-CB. Congener 2,3,5,6-CB also stimulated the onset of Aroclor dechlorination but not as quickly as 2,3,4,5-CB. In addition, the overall dechlorination of Aroclor 1260 was enhanced more by the presence of 2,3,4,5-CB than by 2,3,5,6-CB (Table 1 and Fig. 1). After 6 months, only a small level of *meta* dechlorination continued in the congener-supplemented cultures and all *ortho* dechlorination had ceased. No biphenyl was detected (by GC-MS) in any of the enrichment cultures, and no PCB dechlorination was observed in sterilized slurries (the total chlorine per biphenyl ± standard deviation of triplicate controls was 6.32 ± 0.01).

Dechlorination of added congeners 2,3,4,5-CB and 2,3,5,6-CB was detected after 20 and 27 days, respectively, and preceded the dechlorination of Aroclor 1260. After 181 days, 67 mol% of 2,3,4,5-CB and 99 mol% of 2,3,5,6-CB were transformed to the same products reported by Berkaw et al. (9). Monochlorobiphenyls were produced, including 26 and 12 mol% of 3-CB and 4-CB, respectively, in cultures incubated with 2,3,4,5-CB and Aroclor 1260 and 1 and 32 mol% of 2-CB and 3-CB, respectively, in enrichment cultures supplied with Aroclor 1260 plus 2,3,5,6-CB. Previous (9) and subsequent studies of BH sediments incubated with 2,3,5,6-CB alone have not resulted in the production of 2-CB. We cannot exclude the possibility that 2-CB, or any other monochlorobiphenyl produced, came from the transformation of Aroclor 1260. However, since we cannot unequivocally determine the source of these monochlorobiphenyls, they are discounted in our overall assessment of Aroclor dechlorination when the supplemental congeners are added. No monochlorobiphenyls were detected in samples from slurry enrichments supplied with Aroclor 1260 alone.

The homolog distribution data for Aroclor 1260 before and after incubation can be found in Table 1. Overall, hexa- to nonachlorobiphenyls decreased by 65, 75, and 88% in incubated cultures supplied with Aroclor 1260 alone, Aroclor 1260 plus 2,3,5,6-CB, and Aroclor 1260 plus 2,3,4,5-CB, respectively, indicating more extensive dechlorination of Aroclor 1260 in

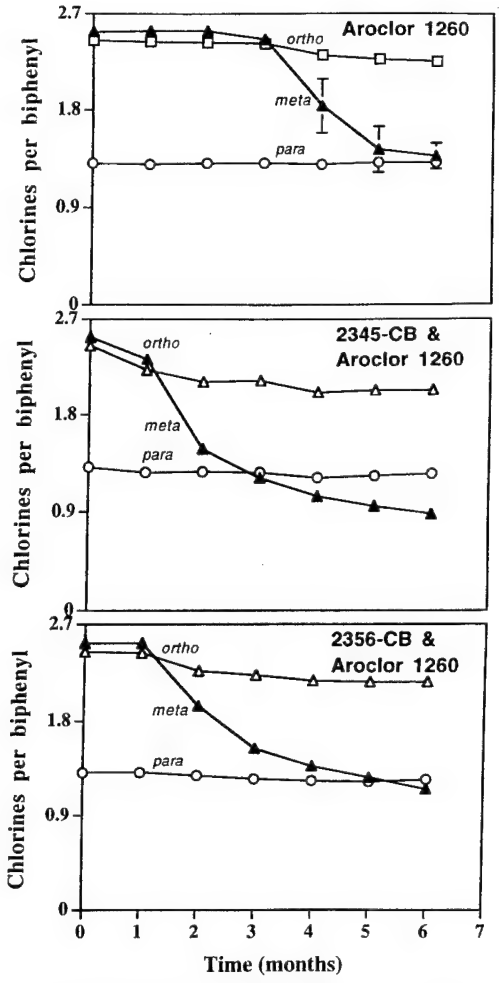

FIG. 1. Chlorine distribution of Aroclor 1260 over the incubation time. Averaged data of triplicate samples are presented. Errors bars indicate standard deviations of triplicate samples; if no error bar is evident, the standard deviation is less than 0.09 and is masked by the symbols.

enrichment cultures supplied with 2,3,4,5-CB. Significant decreases were seen in all of the major hexa- and heptachlorobiphenyls, e.g., 2,3,6-2,4,5-CB, 2,4,5-2,4,5-CB, 2,3,4-2,4,5-CB, 2,3,5,6-2,4,5-CB, 2,3,4,5-2,3,6-CB, 2,3,4,5-2,4,5-CB, and 2,3,4,5-2,3,4-CB. Large increases occurred in tri- and tetrachlorobiphenyls such as 2,4-3-CB, 2,4-3,5-CB, 2,4-2,4-CB, 2,4-2,5-CB, and 2,4-2,6-CB. Small changes in the concentration of pentachlorobiphenyls may indicate an intermediary role for these homologs.

Chlorine distribution of Aroclor 1260 over time indicated that *meta* dechlorination was predominant but was accompanied by a significant, yet more moderate, level of *ortho* dechlorination (Fig. 1 and Table 1). After 181 days, 45 to 65% of the *meta* chlorines and 9 to 18% of the *ortho* chlorines had been removed depending upon congener supplementation. Only a slight decrease of *para* chlorines was observed, although significant *para* dechlorination of 2,3,4,5-CB resulted in cultures supplied with Aroclor 1260 and 2,3,4,5-CB. Comparisons of the congener distributions (± standard deviations) for incubations with Aroclor 1260 alone, Aroclor 1260 plus 2,3,4,5-CB, and Aroclor 1260 plus 2,3,5,6-CB are given in Table 2. Figure 2 presents the data for the 2,3,4,5-CB-supplemented cultures in graphical form, including a difference plot. The data dem-

TABLE 1. Homolog distribution and chlorine distribution of Aroclor 1260 after 181 days of incubation

Homolog or chlorine	Distribution[a]			
	Aroclor 1260 at 0 day	After 181 days		
		Aroclor 1260	Aroclor 1260 + 2,3,4,5-CB	Aroclor 1260 + 2,3,5,6-CB
PCB homologs[b]				
Dichlorobiphenyls	0.10 ± 0.00	0.19 ± 0.03	4.15 ± 0.56	0.98 ± 0.52
Trichlorobiphenyls	0.71 ± 0.01	3.81 ± 0.55	14.87 ± 0.42	5.71 ± 0.80
Tetrachlorobiphenyls	1.42 ± 0.03	53.36 ± 4.53	60.01 ± 1.35	61.67 ± 1.30
Pentachlorobiphenyls	10.81 ± 0.16	11.35 ± 0.55	10.43 ± 0.52	9.84 ± 0.14
Hexachlorobiphenyls	45.65 ± 0.13	12.64 ± 2.66	4.60 ± 1.42	13.54 ± 1.91
Heptachlorobiphenyls	35.61 ± 0.18	14.33 ± 2.55	4.17 ± 1.17	5.90 ± 1.22
Octachlorobiphenyls	5.21 ± 0.09	3.84 ± 0.38	1.36 ± 0.26	1.91 ± 0.31
Nonachlorobiphenyls	0.49 ± 0.01	0.48 ± 0.01	0.41 ± 0.03	0.45 ± 0.01
Chlorines				
ortho	2.49 ± 0.00	2.26 ± 0.02	2.04 ± 0.01	2.19 ± 0.03
meta	2.55 ± 0.00	1.39 ± 0.12	0.89 ± 0.07	1.15 ± 0.05
para	1.27 ± 0.00	1.28 ± 0.01	1.23 ± 0.01	1.23 ± 0.01
Total	6.31 ± 0.01	4.93 ± 0.15	4.16 ± 0.08	4.57 ± 0.07

[a] All data are means of triplicate determinations ± standard deviations. Data for PCB homologs are in moles percent, and data for chlorines are per biphenyl.
[b] No mono- or decachlorobiphenyls were detected.

onstrate that *meta* dechlorination led to substantial increases in 2,4-2,4-CB, 2,4-2,5-CB, and 2,4-2,6-CB and decreases in 2,4,5-2,5-CB, 2,3,6-2,4,5-CB, 2,4,5-2,4,5-CB, 2,3,4-2,4,5-CB, 2,3,4,5-2,3,4-CB, and 2,3,4,5-2,4,5-CB under all conditions. These changes were similar to results reported previously (5, 21). However, 2,3,5,6-2,4-CB, which increased during *meta* dechlorination of Aroclor 1260 residue in Woods Pond sediment (5), was not detected after 6 months of incubation.

ortho dechlorination was evident in all enrichment cultures by the appearance of dechlorination products 2,4-3,5-CB, 2,5-3,5-CB, 2,4-3-CB, and 2,5-3-CB, which are not in Aroclor 1260 (16, 17). Both 2,4-3,5-CB and 2,5-3,5-CB were identified by GC-MS analysis (see Materials and Methods). Although 2,4-3,5-CB and 2,5-3,5-CB could be products of *meta* or *para* dechlorination rather than *ortho* dechlorination, the quantity of substrate congener for such reactions is far less than the amounts of 2,4-3,5-CB and 2,5-3,5-CB observed. As reported by Frame et al. (16), congeners in Aroclor 1260 which were transformed to 2,4-3,5-CB or 2,5-3,5-CB exclusively by *meta* and *para* dechlorination are 2,3,4-3,4,5-CB (0.02 mol%), 2,4,5-3,4,5-CB (0.21 mol%), and 2,3,4,5-3,4,5-CB (0.08 mol%). Far greater than 1.0 mol% each of 2,4-3,5-CB and 2,5-3,5-CB remained in all of our enrichment cultures after 6 months (Fig. 2). However, even higher levels (3.52 to 8.37 mol% of 2,4-3,5-CB and 1.86 to 4.12 mol% of 2,5-3,5-CB) were present in the slurries at earlier times in the experiment. These levels exceed the combined totals of the substrate congeners by more than an order of magnitude. Therefore, the majority of the observed 2,4-3,5-CB and 2,5-3,5-CB in our enrichment cultures is due to *ortho* dechlorination of Aroclor 1260. As the levels of 2,4-3,5-CB and 2,5-3,5-CB declined, corresponding increases in 2,4-3-CB and 2,5-3-CB, which are not present in Aroclor 1260, were observed. The presence of both 2,4-3-CB and 2,5-3-CB was further supported by GC-MS analysis showing the presence of a molecular ion of *m/z* 258 for both of these products. The existence of these trichlorobiphenyls confirms the *ortho* dechlorination that produced 2,4-3,5-CB and 2,5-3,5-CB.

The formation of the non-*ortho*-chlorinated biphenyls 3-3-CB, 3-4-CB, and 3,5-3-CB (Table 2 and Fig. 2) was observed in sediment slurries incubated with Aroclor 1260 plus 2,3,4,5-CB or 2,3,5,6-CB. In addition, 3,5-4-CB was produced in cultures incubated with only Aroclor 1260 and was further dechlorinated in the other cultures. Since none of these congeners are present in Aroclor 1260 (16, 17), they must be products of *ortho* dechlorination because all congeners in virgin Aroclor 1260 contain at least one *ortho* chlorine. 3-4-CB coelutes with 3,4-CB, which is a potential dechlorination product of 2,3,4,5-CB, but we have not observed the formation of 3,4-CB in incubations with only 2,3,4,5-CB. Therefore, 3-4-CB is most likely the result of Aroclor dechlorination. Due to our discount of monochlorobiphenyl production, we do not know whether the non-*ortho* congeners were further dechlorinated to monochlorobiphenyls. Conversely, we observed *ortho*-only-chlorinated congener 2,6-2,6-CB at a low mole percent but no 2,6-2-CB or 2,6-CB/2-2-CB was detected, indicating that dechlorination of 2,6-2,6-CB did not occur in our enrichment cultures.

DISCUSSION

***meta* dechlorination of Aroclor 1260.** Extensive *meta* dechlorination of Aroclor 1260 in BH sediment resulted in significant decreases of PCBs with 2,3,4-, 2,4,5-, 2,3,4,5-, and 2,3,4,6-chlorophenyl groups and corresponding increases in 2,4- and 2,4,6-chlorophenyl groups. These products are the same as those found in Aroclor 1260-contaminated freshwater sediments that have been exposed to dechlorination Process N (2, 7, 21, 31). Process N is characterized by an almost exclusive loss of flanked *meta* chlorines (5, 21). No unflanked *meta* dechlorination of Aroclor 1260 has been reported. In addition to Process N, we observed unflanked *meta* dechlorination of PCBs (e.g., 2,4-3,5-CB→2,4-3-CB and 2,5-3,5-CB→2,5-3-CB) with our enrichment cultures. We suspect that this is primarily due to the *ortho* dechlorination preceding the unflanked *meta* dechlorination in our enrichments. Quensen et al. (21) reported a 19% decrease in the *meta* and *para* chlorines of freshly added Aroclor 1260 with anaerobic microorganisms eluted from Silver Lake after a 19-week incubation. Alder et al. (2) demonstrated a 30% removal of *meta* and *para* chlorines from freshly added Aroclor 1260 with PCB-contaminated sediment from Silver Lake after an 11-month incubation. In comparison to the aforementioned investigations, our results exhibited more extensive *meta* dechlorination (up to 65 mol%) in a relatively

TABLE 2. Changes in PCB congeners of Aroclor 1260 after 181 days of incubation[a]

DB-1 peak no.	PCB congener(s)	Mol% of total PCBs			
		Aroclor 1260 at 0 day	After 181 days		
			Aroclor 1260	Aroclor 1260 + 2,3,4,5-CB	Aroclor 1260 + 2,3,5,6-CB
7	2-3-		0.01 ± 0.02	0.97 ± 0.69	
8	2,3-; 2-4-	0.10 ± 0.00	0.18 ± 0.01	0.32 ± 0.01	0.30 ± 0.02
10	2,6-2-		0.04 ± 0.01	0.08 ± 0.06	
12	3-3-			0.99 ± 0.08	0.12 ± 0.17
13	3,4-; 3-4-			1.80 ± 0.13	0.56 ± 0.37
14	2,5-2-	0.14 ± 0.00	0.49 ± 0.05	1.13 ± 0.02	1.23 ± 0.18
15	2,4-2-	0.05 ± 0.00	0.17 ± 0.02	0.44 ± 0.02	0.37 ± 0.07
17	2,3-2-; 2,6-4-	0.06 ± 0.00	1.02 ± 0.17	1.07 ± 0.19	0.50 ± 0.18
19	2,6-2,6-		1.68 ± 0.31	1.51 ± 0.37	
21	2,5-3-		0.11 ± 0.02	2.83 ± 0.19	0.80 ± 0.26
22	2,4-3-		0.13 ± 0.06	6.96 ± 0.16	1.52 ± 0.38
23	2,5-4-	0.16 ± 0.00	0.29 ± 0.02	0.67 ± 0.03	0.51 ± 0.08
24	2,4-4-	0.10 ± 0.00	1.13 ± 0.14	1.03 ± 0.08	0.55 ± 0.11
25	2,5-2,6-	0.08 ± 0.00	3.06 ± 0.21	4.01 ± 0.20	3.07 ± 0.35
26	2,4-2,6-		9.13 ± 0.63	10.17 ± 0.77	4.77 ± 2.15
28	3,5-3-			0.36 ± 0.05	0.11 ± 0.02
29	2,3-2,6-		0.11 ± 0.00	0.11 ± 0.01	0.15 ± 0.02
30	3,5-4-		0.37 ± 0.09	—[c]	0.06 ± 0.01
31	2,5-2,5-	0.58 ± 0.01	1.30 ± 0.07	2.21 ± 0.06	5.08 ± 0.28
32	2,4-2,5-	0.08 ± 0.00	7.92 ± 0.58	11.05 ± 0.17	14.50 ± 0.16
33	2,4-2,4-		20.11 ± 1.96	25.65 ± 0.85	24.51 ± 0.97
37	2,4,6-2,6-; 2,3-2,5-	0.04 ± 0.00	0.44 ± 0.08	1.62 ± 0.17	0.77 ± 0.15
38	2,3-2,4-		0.16 ± 0.01	0.19 ± 0.08	0.54 ± 0.09
39	2,5-3,5-[b]; 2,3,6-4-	0.14 ± 0.00	2.25 ± 0.12	1.92 ± 0.08	1.86 ± 0.18
40	2,4-3,5-		6.21 ± 0.98	2.45 ± 0.12	3.32 ± 0.19
43	2,4,6-2,5-		1.05 ± 0.11	1.50 ± 0.03	1.42 ± 0.05
44	2,4,6-2,4-		3.81 ± 0.63	5.23 ± 0.46	2.60 ± 0.60
46	2,3,5-2,6-		0.74 ± 0.09		0.01 ± 0.01
47	2,5-3,4-	0.06 ± 0.04	0.35 ± 0.08		1.54 ± 0.18
48	2,4-3,4-[b]; 2,3,6-2,5-	3.32 ± 0.05	0.87 ± 0.20	0.70 ± 0.05	3.54 ± 0.86
49	2,3,6-2,4-		1.08 ± 0.02	0.49 ± 0.10	0.70 ± 0.06
50	2,3-3,4-; 2,3,4-4-	0.18 ± 0.00			
51	2,3,6-2,3-; 2,3,5-2,5	0.32 ± 0.00	0.11 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
53	2,4,5-2,5-; 2,3,5-2,4-	4.09 ± 0.17	0.94 ± 0.21	0.20 ± 0.02	0.38 ± 0.03
54	2,4,5-2,4-	0.13 ± 0.09	1.25 ± 0.09	0.47 ± 0.03	0.74 ± 0.05
55	2,4,6-3,4-; 2,3,6-2,4,6-		1.07 ± 0.05	1.43 ± 0.21	1.96 ± 0.13
57	2,4,5-2,3-	0.13 ± 0.00	0.20 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
58	2,3,4-2,5-	0.35 ± 0.01	0.03 ± 0.00		
60	2,3,6-2,3,6-	1.65 ± 0.01	0.52 ± 0.21	0.77 ± 0.33	2.60 ± 0.49
61	2,3,6-3,4-	1.72 ± 0.02	0.35 ± 0.06		
62	2,4,5-2,4,6-		0.33 ± 0.05	0.58 ± 0.29	1.93 ± 0.43
64	2,3,5,6-2,5-	3.47 ± 0.03	0.69 ± 0.14	0.13 ± 0.03	0.23 ± 0.05
65	2,3,5-2,3,6-	1.07 ± 0.01	0.34 ± 0.08	0.08 ± 0.02	0.13 ± 0.04
66	2,3,4,6-2,5-	0.57 ± 0.01			
69	2,4,5-3,4-; 2,3,6-2,4,5-	9.90 ± 0.04	2.23 ± 0.54	0.68 ± 0.37	5.34 ± 1.46
71	2,3,5,6-2,3-	0.37 ± 0.00	0.05 ± 0.01	0.01 ± 0.00	0.02 ± 0.00
72	2,3,4,6-2,3-; 2,3,5-2,3,5-	0.14 ± 0.00	0.08 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
73	2,3,5-2,4,5-	1.33 ± 0.01	0.64 ± 0.10	0.27 ± 0.03	0.37 ± 0.04
74	2,3,4-3,4-; 2,3,4-2,3,6-	3.28 ± 0.00	0.47 ± 0.10	0.08 ± 0.02	0.12 ± 0.03
75	2,4,5-2,4,5-	9.74 ± 0.01	2.85 ± 0.59	0.73 ± 0.15	1.31 ± 0.22
77	2,3,4,5-2,5-	2.90 ± 0.03	0.60 ± 0.11	0.07 ± 0.04	0.13 ± 0.05
78	2,3,5,6-2,3,6-	2.00 ± 0.03	0.83 ± 0.18	0.28 ± 0.04	0.40 ± 0.07
80	2,3,4,5-2,4-; 2,3,4-2,3,5-; 2,3,4,6-2,3,6-	0.87 ± 0.00	0.23 ± 0.05	0.05 ± 0.01	0.09 ± 0.02
82	2,3,4-2,4,5-; 2,3,5,6-3,4-; 2,3,6-3,4,5-	9.94 ± 0.03	2.65 ± 0.51	0.61 ± 0.17	0.93 ± 0.19
83	2,3,4,6-3,4-	0.23 ± 0.00	0.05 ± 0.01		
85	2,3,5,6-2,3,5-	0.69 ± 0.01	0.20 ± 0.04	0.03 ± 0.01	0.05 ± 0.01
87	2,3,4,6-2,3,5-	0.18 ± 0.01	0.08 ± 0.01	0.01 ± 0.00	0.03 ± 0.01
88	2,3,5,6-2,4,5-	5.15 ± 0.04	2.01 ± 0.36	0.50 ± 0.12	0.75 ± 0.17
90	2,3,4,6-2,4,5-	2.00 ± 0.02	0.65 ± 0.12	0.13 ± 0.03	0.22 ± 0.05
91	2,4,5-3,4,5-	0.93 ± 0.06	0.52 ± 0.19	0.10 ± 0.03	0.14 ± 0.03
92	2,3,4,5,6-2,5-	0.53 ± 0.00	0.18 ± 0.03	0.04 ± 0.01	0.07 ± 0.01
93	2,3,4,5-2,3,6-	4.94 ± 0.01	1.81 ± 0.33	0.68 ± 0.31	0.70 ± 0.14
94	2,3,5,6-2,3,4	2.39 ± 0.01	0.81 ± 0.16	0.19 ± 0.05	0.29 ± 0.06
95	2,3,4,5-3,4-; 2,3,4,6-2,3,4-	1.82 ± 0.06	0.71 ± 0.12	0.20 ± 0.05	0.12 ± 0.03
96	2,3,4-3,4,5-; 2,3,5,6-2,3,5,6-	0.32 ± 0.01	0.12 ± 0.02		0.04 ± 0.03
99	2,3,4,6-2,3,5,6-	0.10 ± 0.01	0.06 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
100	2,3,4,5-2,3,5-	0.72 ± 0.00	0.33 ± 0.05	0.10 ± 0.03	0.15 ± 0.03
102	2,3,4,5-2,4,5-	9.98 ± 0.06	4.87 ± 0.82	1.32 ± 0.32	2.03 ± 0.40

Continued on following page

TABLE 2—*Continued*

DB-1 peak no.	PCB congener(s)	Mol% of total PCBs			
		Aroclor 1260 at 0 day	After 181 days		
			Aroclor 1260	Aroclor 1260 + 2,3,4,5-CB	Aroclor 1260 + 2,3,5,6-CB
103	2,3,5,6-3,4,5-	0.48 ± 0.01	0.35 ± 0.04	0.13 ± 0.01	0.18 ± 0.05
104	2,3,4,6-3,4,5-	0.20 ± 0.00	0.06 ± 0.01	0.02 ± 0.01	0.03 ± 0.00
105	2,3,4,5,6-2,3,6-	0.30 ± 0.02	0.10 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
106	2,3,4,5-2,3,4-	3.72 ± 0.02	1.55 ± 0.25	0.48 ± 0.10	0.68 ± 0.14
107	2,3,4,5,6-3,4-	0.67 ± 0.02	0.54 ± 0.09	0.18 ± 0.04	0.25 ± 0.04
109	2,3,4,5-2,3,5,6-	1.50 ± 0.01	0.92 ± 0.12	0.30 ± 0.07	0.43 ± 0.08
110	2,3,4,5-2,3,4,6-; 2,3,4,5,6-2,4,5-	1.35 ± 0.05	0.89 ± 0.11	0.21 ± 0.06	0.31 ± 0.07
111	2,3,4,5-3,4,5-	0.20 ± 0.00	0.13 ± 0.01	0.05 ± 0.01	0.07 ± 0.01
112	2,3,4,5,6-2,3,4-	0.64 ± 0.00	0.50 ± 0.05	0.19 ± 0.03	0.27 ± 0.03
113	2,3,4,5,6-2,3,5,6-	0.16 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
115	2,3,4,5-2,3,4,5-	1.26 ± 0.04	1.30 ± 0.07	0.61 ± 0.09	0.81 ± 0.08
117	2,3,4,5,6-2,3,4,5-	0.48 ± 0.01	0.49 ± 0.01	0.40 ± 0.02	0.44 ± 0.01

[a] All data are the means of triplicates ± standard deviations.
[b] Not detected in Aroclor 1260 (16, 17).
[c] 3,5-4-CB was detected at 0.2 mol% after 2 to 4 months of incubation.

shorter period of time. This further demonstrates the potential for reductive dechlorination of haloaromatic compounds in estuarine sediments.

***ortho* dechlorination of Aroclor 1260.** At least six distinct microbial dechlorination processes can be recognized as occurring in various contaminated sediments on the basis of congener selectivity and the products observed in situ and in laboratory studies (5, 10, 12). In all previous reports, PCBs are dechlorinated only by loss of *meta* and/or *para* chlorines. Here we have demonstrated the occurrence of *ortho* dechlorination of Aroclor 1260 added to BH sediment. The results suggest that such activity could play a role in the bioremediation of Aroclors in marine and estuarine sediments. This is the first confirmed report of *ortho* dechlorination of PCB mixtures, although *ortho* dechlorination of single congeners has also been reported (9, 19, 28–30). Maximal chlorine removal appears to require the complementary action of two or more dechlorination processes (5, 21). For example, in Process N (flanked *meta* dechlorination of Aroclor 1260), elevated amounts of 2,3,5,6-2,4-CB are produced by *meta* dechlorination of 2,3,5,6-2,4,5-CB and 2,3,5,6-2,3,4-CB, and 2,3,5,6-chlorophenyl substituents are recalcitrant in Aroclor 1260 (5). However, no 2,3,5,6-2,4-CB was observed in our slurry enrichments because the 2,3,5,6-2,4,5-CB and 2,3,5,6-2,3,4-CB were *ortho* and *meta* dechlorinated to 2,4-3,5-CB and 2,4-3-CB. Thus, the combination of *ortho* dechlorination plus flanked and unflanked *meta* dechlorination resulted in more dechlorination than that produced by the flanked *meta* dechlorination of Process N.

Specificity of *ortho* dechlorinating activity. A modest amount of *ortho* dechlorination was observed in comparison to the amounts of *meta* dechlorination in all of our enrichment cultures. We hypothesize that the moderate *ortho* dechlorination of the Aroclor in our enrichments is dependent on the specificity of *ortho* dechlorinating microorganisms in BH sediment. Previously, we reported on the *ortho* dechlorination of a few single PCB congeners (9). Among those congeners, ~99 mol% of 2,3,5-CB, ~20 mol% of 2,3,6-CB, and ~92 mol% of 2,3,5,6-CB were *ortho* dechlorinated. In that report, no *ortho* dechlorination was observed in BH sediment incubations supplied with 2-CB, 2,3-CB, 2,4-CB, 2,5-CB, 2,6-CB, 2,4,6-CB, 2,6-2,6-CB, or 2,3,4,5-CB over a 6-month period. However, after incubating the cultures for more than a year, we have now observed the *ortho* dechlorination of 2,4-CB and 2,4,6-CB to 4-CB and a small amount of 2,6-2,6-CB to 2,6-2-CB in enrichment cultures supplied with these single congeners (data not shown). Others have also reported on the *ortho* dechlorination of unflanked *ortho* chlorines after extended incubation (29, 30). These results indicate that although some unflanked *ortho* dechlorination will occur after extended incubation, the *ortho* dechlorinators in BH sediment favor removal of flanked *ortho* chlorines, with the exception of 2,3-CB and 2,3,4,5-CB. In Aroclor 1260 (16), only 12 mol% of the congeners bear 2,3,5- and 2,3,5,6-chlorophenyl groups. Congeners carrying 2,3,6-chlorophenyl groups (14 mol%) and 2,3,4-, 2,4,5-, and 2,3,4,5-chlorophenyl groups (55 mol%) are far more prevalent. Therefore, relatively smaller amounts of PCBs with 2,3,5- and 2,3,5,6-substitutions in Aroclor 1260 may explain why only moderate levels of *ortho* dechlorination of Aroclor 1260 were observed in our BH sediment enrichment cultures. Based on the results presented here and previously with single congeners (9), we propose pathways for the dechlorination of Aroclor 1260 to the major *ortho* dechlorination products observed in these experiments (Fig. 3).

Although we did not perform controlled experiments, we have observed in general that PCB dechlorination is more stable, i.e., more extensive and with shorter lag times, when sediments are stored anaerobically at room temperature (20 to 22°C) than at 4°C. K. R. Sowers has also observed this with the storage of sediments from several sites used for methanogenic enrichments. Room temperature storage is a possible explanation for why *ortho* dechlorination could be activated even after the sediment had been stored for 14 months. Other laboratory studies have revealed that a prolonged storage time of sediment at 4 to 7°C increased the incubation time required to transform 50% of the substrate tested for chlorophenol dechlorination (32) and changed the PCB dechlorination primed by 4-bromobenzoate (25). However, it is also important to note that room temperature storage of an estuarine or marine sediment does not ensure the development of *ortho* dechlorination. Using identical storage and enrichment conditions, we have not been able to enrich for *ortho* dechlorination with three of five Charleston Harbor (Charleston, S.C.) sediments and one sediment from the middle of the Chesapeake Bay near the mouth of the Potomac River. *meta* or *para* dechlorination developed with each of these sediments (data not shown). Therefore, something specific to the site, perhaps the microbial

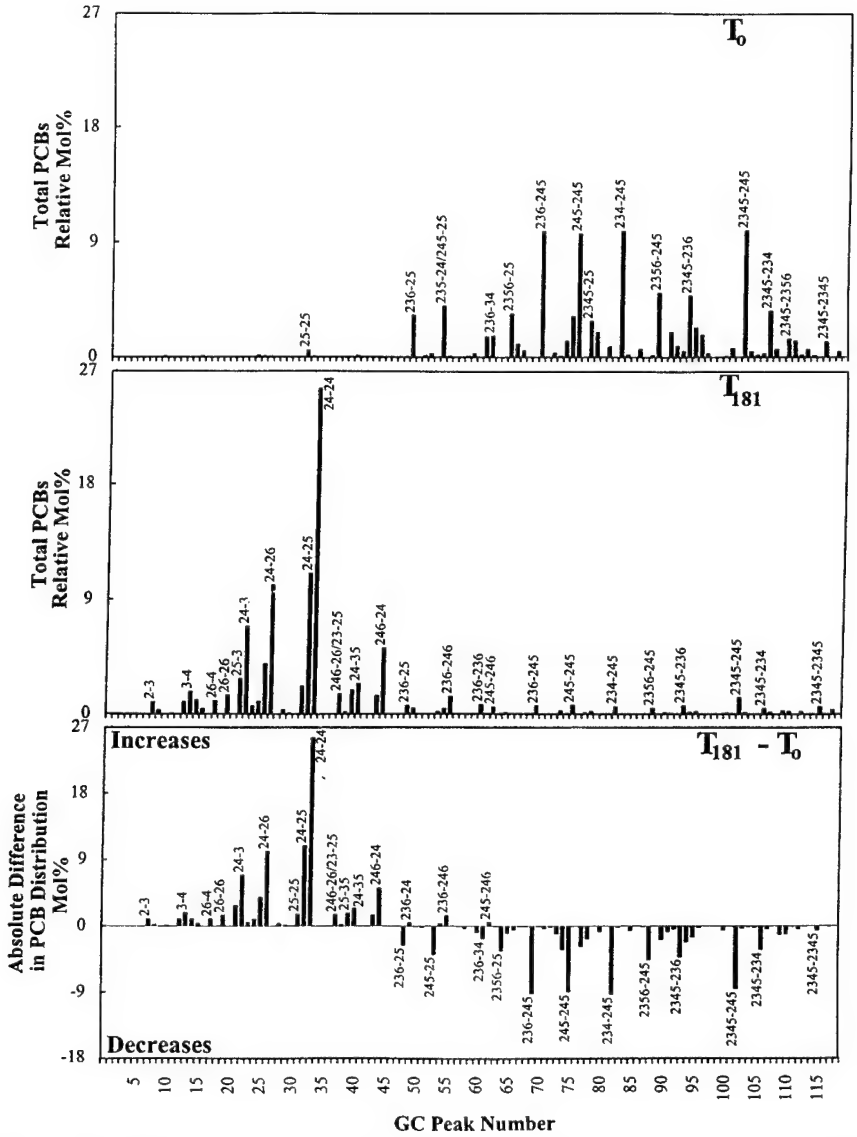

FIG. 2. Congener distribution of Aroclor 1260 at time zero (T_0) and after 181 days (T_{181}) of incubation in incubated cultures supplied with Aroclor 1260 and 2,3,4,5-CB. Averaged data of triplicate samples are presented.

population, is more critical for the development of *ortho* dechlorination than storage temperature.

Effect of added 2,3,4,5-CB and 2,3,5,6-CB on dechlorination of Aroclor 1260. Microbial PCB dechlorination of Aroclor 1260 residue can be primed by the addition of elevated concentrations (200 to 500 μM) of certain PCB congeners (3, 5, 7, 31). Bedard and colleagues (3, 7) found that they could stimulate Process N and Process P (flanked *para* dechlorination) of Aroclor 1260 residue in Woods Pond sediment by the addition of 2,3,4,5,6-CB and 2,5-3,4-CB, respectively. The addition of 2,3,4,6-CB also stimulated Process N, Process P, and Process LP (unflanked *para* dechlorination) of Aroclor 1260 residue in Woods Pond sediment and led to a 34% decrease in *meta* and *para* chlorines after 12 months of incubation at 25°C (31). Our results indicate that the addition of single PCB congeners (2,3,4,5-CB and 2,3,5,6-CB) stimulates *meta* and *ortho* dechlorination of Aroclor 1260 in these sediments (shorter lag time and more extensive dechlorination), further supporting the

2,3,5,6-2,5-CB^a/2,3,5-2,3,6-CB^a → 2,3,5-2,5-CB^a → 2,5-3,5-CB^b → 2,5-3-CB^b → 2-3-CB^b

2,3,5,6-2,3,4,5-CB^a → 2,3,5,6-2,4,5-CB^a/2,3,5,6-2,3,4-CB^a → 2,3,5-2,4,5-CB^a → 2,4,5-3,5-CB^c → 2,4-3,5-CB^b → 2,4-3-CB^b

FIG. 3. Proposed pathway of *meta* and *ortho* dechlorination of PCB congeners in Aroclor 1260 to produce 2,4-3,5-CB, 2,5-3,5-CB, 2,4-3-CB, 2,5-3-CB, and 2-3-CB. Superscript *a* designates a decreased congener after incubation; superscript *b* designates a congener appearing after incubation; superscript *c* designates a proposed intermediate, which could not be identified due to its coelution with 2,4,5-2,4-CB.

hypothesis that anaerobic bacteria derive energy by donating electrons to halogenated biphenyls (10, 12, 22).

In summary, anaerobic microorganisms in BH estuarine sediments reductively dechlorinate Aroclor 1260. The dechlorination of Aroclor 1260 is extensive and results in removal of *meta* and *ortho* chlorines. The addition of single PCB congeners stimulates the *meta* and *ortho* dechlorination of Aroclor 1260. Reviewed together, these results demonstrate that the biocatalytic capability of anaerobic microorganisms to reductively dechlorinate PCBs is broader than previously realized. Such activity could prove useful in the bioremediation of PCBs and awaits testing with PCB-contaminated (aged) sediments.

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Poster no. 66

Differential RFLP patterns of PCR-amplified 16S rDNA from anaerobic PCB-dechlorinating estuarine and marine sediment enrichments.

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Para-, meta- and *ortho*-dechlorination activities of individual polychlorinated biphenyl congeners (PCBs) by sediments from Baltimore Harbor have been maintained throughout sequential transfers of enrichment cultures. However, microorganisms responsible for dechlorination have not yet been identified by standard isolation techniques. In order to identify PCB - dechlorinating microorganisms, a procedure was developed for the isolation and PCR amplification of DNA encoding 16S rRNA from marine and estuarine enrichments containing sediments with high concentrations of humic acids. Enrichment slurries were lysed with glass beads in phosphate burrer containing Denhardt's solution and the DNA was extracted with phenol-chloroform. Humic acids associated with DNA were removed with insoluble polyvinylpyrrolidone, extracted from a low-melt agarose gel containing soluble polyvinylpyrrolidone, then amplified by PCR using universal oligonucleotide primers. DNAs encoding 16S rRNA from Archaea (*Methanosarcina thermophila*), Bacteria (*Escherichia coli*), and Eucarya (*Morone saxatilis*) are recovered and amplified from as few as 10^2 cells in sediment slurry. Differential RFLP patterns from PCR generated 16S rDNA are shown for enrichments that *para-, meta-* and *ortho*-dechlorinate 2,3,4,5-PCB, as well as cultures that exhibit *para-* or *ortho*-dechlorination of 2,3,4,5-PCB and 2,3,5,6-CB, respectively. Initial analyses of the gene sequences from representative RFLP patterns indicate that this approach is effective for discrimination of mixed rDNA populations in PCB-dechlorinating enrichments that are up to 98% homologous.

Berkaw, M., L. Cutter, K.R. Sowers, and H.D. May. 1996. Site-dependent *ortho-, meta-, and para* -dechlorination of PCBs by anaerobic estuarine and marine sediments enrichments. 5th European Marine Microbiology Symposium, Bergen, Norway.

Poster no. 67

Site-Dependence of *ortho*, *meta*, and *para* Dechlorination of PCBs by Anaerobic Estuarine and Marine Sediment Enrichments.

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Anaerobic dechlorination of polychlorinated biphenyls (PCBs) was observed in enrichments with estuarine and marine sediments collected along the eastern seaboard of the United States (Chesapeake Bay and Charleston Harbor). The enrichments were incubated in the dark at 30°C under obligately anaerobic conditions in estuarine and marine media containing short chain fatty acids as potential donors of carbon and electrons. The PCBs were extracted in ethyl acetate, passed over copper:florisil, and analyzed by gas chromatography (electron capture and mass spectral detection). The dechlorinations developed within 1 month and included removal of *ortho*, *meta*, and *para* chlorines from several PCB congeners. The specific type of reductive dechlorination observed was site-dependent. An unusual removal of *ortho* chlorines was detected in enrichments with sediment from 3 of the 7 sites examined. Sediments from 2 of the 7 sites have only expressed *para* dechlorination. Sequential transfer in minimal medium containing a single PCB congener (2,3,5,6-CB) has resulted in the selective enrichment of *ortho* dechlorination. Selective enrichment of other specific activities is being pursued with alternative single congeners with the intent of isolating and characterizing the PCB-dechlorinating microorganisms.

contains 2,3,4,2',5'-CB, 3,4,5,2'-CB; 3,4,5,2',5'-CB; 3,5,3',5'-CB; 2,4,2',4'-CB; 2,4,4'-CB and 3,4,5,3'-CB. Equal rates for depletion of biphenyl and formation of *cis*-biphenyl 2,3-dihydrodiol confirmed biphenyl 2,3-dioxygenase activity was responsible for depletion of CBs. In addition, chlorinated *cis*-dihydrodiols were detected as oxidation products by gas chromatography-mass spectrometry. Analysis of the product formed from 2,5,3',4'-CB by ^{1}H-nuclear magnetic resonance spectrometry showed that molecular oxygen was incorporated at the 3,4-position of the 2,5-dichloro-substituted ring. The data suggest that the position rather than the number of chlorine ring substituents is a major factor determining the specificity of biphenyl 2,3-dioxygenase for CBs.

Q-31. Evolution of a Pathway for Chlorobenzene Metabolism in a Contaminated Ecosystem

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Ecosystems contaminated with chlorobenzene contain bacteria able to grow on chlorobenzene whereas uncontaminated ecosystems do not. The pathway for metabolism of chlorobenzene is uncommon among aerobic bacteria. Previous work has shown that recombination between separate gene clusters that encode an aromatic ring dioxygenase and the enzymes that catalyze chlorocatechol degradation can produce a functional pathway. The pathway for chlorobenzene degradation could have evolved once and been dispersed or, alternatively, it could have evolved from indigenous bacteria in each contaminated ecosystem. We designed experiments to distinguish between the two possibilities. A CB-degrading organism, *Ralstonia* sp. JS705, with a unique CB-degradation pathway organization was isolated from contaminated groundwater at Kelly Air Force Base, Texas. DNA probes and primers derived from the chlorobenzene and chlorocatechol dioxygenase genes of JS705 were used to screen total DNA as well as a variety of bacteria isolated from wells within and outside the CB plume. DNA from independently isolated CB-degrading bacteria hybridized with both probes as did total DNA isolated from CB-contaminated wells. Southern hybridizations revealed that CB-degrading isolates from the site contained CB-degradative genes and 16S rDNA genes closely related to those of JS705. Physiological characterizations using Biolog plates indicated minor differences among isolates. The sequence of a 500 bp section of the chlorobenzene dioxygenase gene of JS705 was identical to that of the benzene and toluene dioxygenase genes in strains isolated from the site for the ability to degrade benzene or toluene but not CB. Genes from CB-degrading strains isolated at other CB-contaminated sites were not closely related to the genes from JS705. The results indicate that the pathway arose once at the site through genetic recombination between an acestral benzene- or toluene-degrading strain and an unknown strain containing the genes for the chlorocatechol degradative pathway.

Q-32. Characterization of Class I Extradiol dioxygenase from a strong PCB degrader Rhodococcus sp. Strain RHA1

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Aromatic-ring-cleavage extradiol dioxygenases are categorized into three classes on the basis of sequence similarity. The three-dimensional structure of 2,3-dihydroxybiphenyl 1,2-dioxygenases (BphCs) from *Pseudomonas* sp. strain KKS102 and *Burkholderia* sp. LB400 revealed that the class II enzymes are composed of two homologous domains (N- and C-terminal domains). On the other hand, the class I enzymes are half the size of class II and class III enzymes and supposed to be composed of a single domain. The class II and class III enzymes are deduced to have evolved by the duplication of a class I enzyme gene followed by the mutation and loss of function. Multiple BphC-isozyme genes seems to be common in Rhodococcal PCB degraders, and include class I enzyme genes such as *R. globerulus* P6 *bphC2* and *bphC3*, and *R. erythropolis* TA421 *bphC2*. The presence of class I enzyme gene homologue (*bphC6*) was suggested in a strong PCB degrader RHA1. To obtain a functional implication of class I enzymes, we characterized the *bphC6* gene and its product BphC6 of *Rhodococcus* sp. RHA1. The cloned *bphC6* encodes 171 amino acid residues and its deduced amino acid sequence showed 85% and 65% identity to *R. globerulus* P6 BphC2 and BphC3, respectively. Only a small amount of BphC6 was produced in *E. coli*, even under the lac promoter, and we employed the His-tagged expression system. The His-tagged fusion BphC6 (H-BphC6) expressed successfully, and purified to homogeneity. The gel-filtration chromatography indicated the homo-dimer or homo-hexamer structures for the native H-BphC6 enzyme. The H-BphC6 was very specific to 2,3-dihydroxybiphenyl, and exhibited faint and little activity toward 3-methylcatechol and catechol, respectively. The RHA1 H-BphC6 may be involved in the metabolism of a biphenyl-related compound.

Q-33. Bioaugmentation for In Situ Treatment of Chlorinated Solvent-Contaminated Groundwater

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A field-scale demonstration of bioaugmentation for TCE remediation was performed by injecting an adhesion-deficient TCE-degrading bacterium, ENV435, into a confined aquifer. The organisms were injected at 6 up-gradient wells, and their migration through the aquifer and degradation of TCE, DCE, and vinyl chloride was measured in a series of monitoring wells over a distance of approximately 40 feet. Hydraulic control within the test plot was maintained by recirculating groundwater from a down-gradient recovery well, through an oxygenation system, and into the 6 up-gradient injection wells. A "control" plot was operated without added bacteria. After 2 injections of organisms, chlorinated ethene concentrations within the test plot were reduced from approximately 2 ppm to less than 50 ppb at some monitoring wells. Furthermore, viable cells of ENV435 were recovered throughout the test plot. No loss of chlorinated ethenes was observed in the control plot.

Q-34. Dichloroethene Biodegradation Under Mn(IV)-Reducing Conditions

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Dichloroethene (DCE), an intermediate product of reductive dechlorination of polychlorinated ethenes, is one of the most common ground-water contaminants in the US and an EPA priority pollutant. Unlike its parent compounds, tetrachloroethene and trichloroethene, which are highly oxidized and readily undergo reductive dechlorination, DCE is relatively reduced and resistant to reductive degradation except under highly reducing, methanogenic conditions. Moreover, due to the oxidized nature of the chlorine substituent, DCE is also resistant to oxidative degradation and, to date, substantial oxidation of DCE has been reported only under aerobic conditions. Mn(IV) oxides are potentially powerful oxidants that are often present in natural ground-water systems. Here we report the first evidence of rapid anaerobic DCE oxidation to CO_2 under Mn(IV)-reducing conditions. These results indicate that oxidative degradation of partially chlorinated solvents, like DCE, can be significant under anoxic conditions and demonstrate the potential importance of Mn(IV) reduction for remediation of chlorinated ground-water contaminants.

Q-35. Two Anaerobic Polychlorinated Biphenyl-Dechlorinating Enrichment Cultures with Different Substrate Specificities

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Two anaerobic polychlorinated biphenyl (PCB)-dechlorinating enrichment cultures were obtained from PCB-free estuarine sediment (Charleston, SC): a 2,3,4,5-tetrachlorobiphenyl (2345-CB) *para*-dechlorinating culture and a 2,3,5,6-tetrachlorobiphenyl (2356-CB) *meta*-dechlorinating culture. These enrichments have been sequentially transferred 8 times in estuarine medium containing 0.1 g% of sediment and 175 mM 2345-CB or 2356-CB. The dechlorination activity of the 2345-CB enrichment culture could also be maintained after sequential transfers in estuarine medium without sediment. The dechlorination activity of the 2345-CB enrichment culture increased in the presence of H_2 or 10 mM fumurate, while addition of 10 mM formate or 10 mM acetate plus 2 mM bromoethanesulfonate enhanced the dechlorination activity of the 2356-CB enrichment culture. The dechlorination activities of these two cultures were inhibited in the presence of 10 mM molybdate or 100 mg streptomycin per ml. In the presence of 100 mg vancomycin per ml, dechlorination activity was observed in the 2345-CB culture, but not in the 2356-CB culture. Experiments with 8 - 11 tested PCB congeners indicated that 2345-CB enrichment culture primarily *para* dechlorinated PCBs tested and 2356-CB enrichment culture only *meta* dechlorinated PCBs. The results suggest that these two PCB-dechlorinating cultures contain different PCB-dechlorinating microorganisms, each with different carbon source and PCB congener specificities.

Q-36. Degradation of 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE) in Soil

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While aerobic transformation of DDE in liquid culture has been previously demonstrated, there are no published reports of DDE transformation in temperate soils. In this study we investigated the ability of a recombinant organism, Pseudomonas acidovorans M3GY, to transform ^{14}C-DDE in soil and soil slurries. The influence of a surfactant, Triton X-100, on DDE transformation in slurries was also studied. The greatest level of transformation (>50 % disappearance) occurred in the unsaturated soil. Although significant transformation occurred in the soil slurries (>35% disappearance), there was no significant difference in transformation when slurries were amended with either 100 or 200 ppm Triton X-100. Total counts of biphenyl-degrading bacteria were similar for both the slurry and the unsaturated treatment, however, lower numbers of M3GY were recovered from the slurry.

Q-37. Development of a Field Application Vector for PCB-Contaminated Soils

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Selected plant-derived terpenes similar to carvone, the principal component of spearmint oil, can induce polychlorinated biphenyl (PCB) biodegradation by Arthrobacter sp. strain B1B. Microcosm studies further demonstrated that repeated application of carvone-induced cells to Aroclor 1242-contaminated soil resulted in 27 percent degradation of total PCB over nine weeks. In an effort to improve the bioavailability and metabolism of soil-bound PCBs, we have developed a field application vector based on growth of Arthrobacter sp. strain B1B on sorbitan trioleate (ST), a commercially available surfactant. Strain B1B grew vigorously in ST medium, and had a half-saturation constant for ST of 95 ppm. A preliminary study found that ST effectively desorbed 22 percent of the total PCB in a contaminated soil after

esses governing MTBE biodegradation.Controlled studies using batch incubations monstrated that MTBE removal by the GAC was a combination of physical sorption d biological degradation. Maximum MTBE removal rates are estimated to be on the er of 4,000 mg MTBE/g GAC/day with an apparent half-saturation constant of proximately 7,000 mg MTBE/L (in the presence of GAC). Removal of MTBE by the C appears to be pH sensitive.Forty-nine bacterial strains were isolated from the GAC enrichment on MTBE and plating on both selective and non-selective media. These lated strains were grouped into nine colony phenotypes. At least two phenotype ups had representative strains that oxidized MTBE. Preliminary analysis suggests t the true half-saturation constant for the pure cultures is several orders of magni- e lower than that observed in the reactor and that the maximum specific MTBE oxi- on rates are low. The significance of theses results to the biological treatment of TBE will be discussed.

315. Acrylamide Degradation by a *Pseudomonas aeruginosa* Strain

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reasing use and indiscriminate discharge of acrylamide and other related amides is aming a serious type of contaminants in soil and water. Higher concentrations of these ides do not degrade rapidly. The purpose of this study was to screen and isolate bac- al strains capable of degrading acrylamide efficiently. We have isolated a strain of *udomonas aeruginosa* from the effluent of an explosive factory which showed excel- t growth with as high as 63 mM acrylamide. Complete inhibition of growth was ved at 90 mM. Our results show that acrylamide is used as the sole sources of car- n and nitrogen for the growth of *P. aeruginosa*. Employing GLC technique, the pri- ry product of acrylamide degradation has been identified as acrylic acid. Another tabolite in the culture filtrate was determined to be ammonia. Formation of acrylic d and ammonia by *P. aeruginosa* revealed close correlation with the disappearance of ylamide from the medium. Enzyme responsible for acrylamide degradation has been ntified as amidase which was inducible in nature. *P. aeruginosa* appears to be a ent degrader of acrylamide and may be employed in bioremediation.

316. Construction of Environmental DNA Libraries and Screening Anaerobic Utilization of 4-Hydroxybutyrate by Recombinant *-herichia coli* Strains

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genetic diversity of the microorganisms in an environment offers interesting portunities to encounter new or unproved genes and gene products for biotechnol- cal purposes. In order to exploit the genetic diversity DNA libraries of several envi- ments were constructed. DNA was extracted from various soil samples by lysis with -salt extraction buffer and extended heating in the presence of SDS. The final ification was performed with the Wizard® Plus Minipreps DNA Purification tem. The purified DNA was partially digested with *Bam*HI or *Sau*3AI, ligated in bescript SK and transformed into *Escherichia coli*.

resulting recombinant *E. coli* strains were screened on tetrazolium indicator plates the utilization of 4-hydroxybutyrate (4-HB): six out of approximately 270,000 es were positive. These clones showed a slower growth rate on 4-HB than *E. coli* 09/pCK1, which harbors the gene encoding 4-HB dehydrogenase from *tridium kluyveri*. Enzymatic analysis revealed 3-HB and 4-HB dehydrogenase ivity in crude extracts of the recombinant *E. coli* strains. The inserts of the plasmids ted from these strains were sequenced. The deduced gene products exhibited no ificant similarity to any other known protein.

317. Characterization of Selective ortho PCB-Dechlorinating richment Cultures by Comparative Sequence Analysis of S rDNA

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richment cultures that selectively ortho-dechlorinate 2,3,5,6-tetrachlorobiphenyl e analyzed by comparative sequence analysis of 16S rDNA genes amplified from d community DNAs in order to identify potential PCB-dechlorinating anaerobes. ulation profiles are presented from enrichments that ortho dechlorinate 2,3,5,6-CB ne presence or absence of sediment. Dechlorination in the presence of fatty acids or tate showed that different carbon sources select for different populations. Population files from enrichments exposed to specific inhibitors (bromoethanesulfonic acid, comycin, and molybdate) demonstrated that highly enriched PCB-dechlorinating erobes could be obtained. In addition, molecular monitoring showed that some ly enriched species found in dechlorinating cultures were absent in inactive cultures n enrichment cultures developed without PCBs. By combining selective enrichment molecular monitoring (SEMM technology), defined ortho-dechlorinating consortia e been established and maintained through sequential transfers.

Q-318. Functional Analysis of the *Pseudomonas syringae rulAB* Determinant in Tolerance to Ultraviolet B (280 to 320 nm) Radiati and Distribution of *rulAB* Among *P. syringae* Pathovars

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The bacterial plant pathogen *Pseudomonas syringae* is adapted to growth and s vival on leaves in the phyllosphere, a habitat which is normally exposed to l doses of natural UV radiation. We recently determined that the indigenous pl mids pPSR1 and pPSR5 from *P. syringae* pv. syringae contained a homolog of *umuDC* mutagenic repair operon termed *rulAB* which functioned in tolerance UVC (254 nm) radiation (Gene [illegible]-81). In this study, we analyzed the role *rulAB* in conferring tolerance to environmentally-relevant levels of UVB radiati both in vitro and in the phyllosphere. We also examined the distribution and U sensitivity of a worldwide collection of *P. syringae* pathovars. We examined differences in survival of *P. syringae* pv. syringae FF5 containing the *rulAB* det minant cloned in pCWS157 and FF5 containing the vector control. Measur doses of UVB radiation were delivered either to cells previously grown in LB br and resuspended in 0.85% NaCl or to populations established from one to fi days in the bean phyllosphere. Our results indicated that the survival FF5(pCWS157) was approximately ten to twenty-fold greater than FF5(vect following irradiation of cell suspensions with a range of UVB doses (750 to 1,1 J m^{-2}). A difference in percent survival of five to ten-fold was observed in comparison of FF5(pCWS157) and FF5(vector) following the irradiation of be phyllosphere populations with a UVB dose of 850 J m^{-2}. This smaller differe was attributed to the ability of a portion of the total FF5 population on bean access sites within bean leaves protected from the UVB dose. Analysis of the U sensitivity (850 J m^{-2} dose) in vitro of a worldwide collection of 64 *P. syring* strains representing 10 pathovars indicated that the most tolerant and most s sitive strains differed in percent survival by approximately 125-fold. We utiliz Southern hybridization with an internal fragment of *rulAB* as a probe to show t 71.9% of the strains contained plasmid homologs of *rulAB* and that only two the pathovars examined (actinidiae and syringae) included strains which did contain *rulAB* hybridizing sequences. Strains which contained *rulAB* sequen were on average 5-50 fold more tolerant of UVB irradiation. Thus, the cloned *syringae rulAB* determinant was shown to confer significant levels of tolerance UVB radiation both in vitro and in the natural habitat (phyllosphere) of the b terium. Also, the phenotype of UVB tolerance and the plasmid-encoded *rul* genes were widely distributed among *P. syringae* pathovars. Our data suggest t tolerance to UVB radiation in *P. syringae* is an important component of ecologi fitness in the phyllosphere.

Q-319. Characterization of Motor oil Utilizing Bacteria from Goucher Pond

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Twenty two bacterial isolates were obtained from oil slicks on Goucher Pond. The b teria were isolated in minimal salt medium with 0.1 to 1% motor oil. Many isola produced fluorescent pigments in King's B medium, which is limited in iron conte Nutritional and metabolic assays indicated that many of these bacteria belong to fluorescent pseudomonads including Pseudomonas chlororaphis, P. fluorescens, putida, and P. viridiflava. One fluorescent isolate had the characteristics Pseudomonas cepacia or P. gladioli which are not known to produce fluorescent p ments. One isolate, which was originally cocultivated with a fluorescent pseudom ad, was identified as Serratia ficaria. Except for S. ficaria, all isolates characteri thus far appear to produce rhamnolipids. Serratia ficaria alone did not survive medium with motor oil as the sole carbon source. Some pseudomonad grew in mo oil as sole carbon source. However, its presence augmented the growth of oil pseudomonads in motor oil.

Q-320. Sulfur Cycling Mediates Calcium Carbonate Geochemistry Modern Marine Stromatolites

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Modern marine stromatolites forming in Highborne Cay, Exumas (Bahamas), co tain microbial mats dominated by *Schizothrix*. Although saturating concent tions of Ca^{2+} and CO_3^{2-} exist, microbes mediate $CaCO_3$ precipitati Cyanobacterial photosynthesis in these stromatolites aids calcium carbonate p cipitation by removal of H^+ through CO_2 use. Photorespiration and exopolyn production predominantly by oxygenic phototrophs fuel heterotrophic activi aerobic respiration (approximately 60 mmol/cm^2.h) and sulfate reduction (S 1.2 mmol SO_4^{2-}/cm^2.h) are the dominant C-consuming processes. Aerobic mic bial respiration and the combination of SR and H_2S oxidation both facilit $CaCO_3$ dissolution through H^+ production. Aerobic respiration consumes m more C on an hourly basis, but diel fluctuating O_2 and H_2 depth profiles indic that overall, SR consumes only slightly less (0.2-0.5) of the primary producti Moreover, due to low O_2 concentrations when SR rates are peaking, reoxidation the H_2S formed is incomplete: both thiosulfate and polythionates are formed. T process of complete H_2S oxidation yields H^+. However, due to a low O_2 conc tration late in the day and relatively high O_2 concentrations early in the follow morning, a two-stage oxidation takes place: first, polythionates are formed fr H_2S, creating alkalinity which coincides with $CaCO_3$ precipitation; secondly, o dation of polythionates to sulfate yields acidity, resulting in dissolution.

Q-139. Aerobic Degradation of Polychlorinated Biphenyls by Boreal Freshwater Sediment Cultures

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We studied the capability of indigenous freshwater sediment microorganisms to degrade polychlorinated biphenyls (PCBs) under aerobic conditions. Sediment samples were collected from a boreal lake (Lake Kernaalanjarvi, Finland) which has been exposed to a minor PCB load for several decades. Typical PCB concentrations in the surface sediment are around 1 mg/kg d.w. and the maximum concentrations do not exceed 15 mg PCBs/kg d.w. Aerobic biphenyl degraders, enriched from the surface sediment samples, were tested for their ability to degrade Aroclor 1242. Initial PCB concentration in batch vial experiments ranged from 25 to 200 mg/L. Aroclor 1242 did not serve as a growth substrate for the enrichment cultures, but was cometabolized in the presence of biphenyl. Selected mono-, di-, and trichlorobiphenyls were degraded resulting in the total degradation of approximately 20% over a period of one week. Congeners with a substitution pattern of either 2,2'-, 2,6-, or 4,4'-, and those carrying more than three chlorine substituents resisted degradation. Biphenyl concentration affected degradation considerably. Trichlorobiphenyls present in Aroclor 1242 were resistant in the absence of biphenyl. Furthermore, the degradation typically ceased after a few days of incubation although biodegradable congeners were still present. This was explained by the depletion of biphenyl. The extent of total PCB degradation was modest. More importantly, we demonstrated that indigenous boreal lake sediment microorganisms, exposed to low-level PCB contamination, have the potential to degrade high concentrations of selected lower chlorinated PCB congeners in Aroclor 1242.

Q-140.

Assessment of *In situ* Anaerobic PCB Dechlorinators in a Contaminated Sediment Consortium.

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A method for assessing the presence of PCB dechlorinating organisms in soils and sediments is essential to understanding intrinsic remediation. Sediment from Lake Medinah, NY was used to establish laboratory microcosms having distinct *meta* and *para* dechlorination activities with regards to 2',3,4-trichlorobiphenyl. DNA from the anaerobic heterotrophic bacteria, chiefly clostridia, from each community was extracted and analyzed by Amplified Ribosomal DNA Restriction Analysis. Oligonucleotide probes based on 16S rRNA genes were designed for the most abundant Operational Taxonomic Unit (OTU A and B) in each microcosm. To detect the presence of OTU-A and OTU-B in sediment samples, the probes were used in dot blot and Southern hybridization studies. Eubacterial and Archaebacterial primers were used to amplify 16S rDNA from the same DNA. Interestingly, there was PCR product with the Archaea primers, suggesting that Archaea, as well as members of the Genus *Clostridium* are involved. Results indicate that the most predominant member of the consortium was detectable and could be used as an indicator for natural attenuation in other sediments.

Q-141.

Reductive Dechlorination of Polychlorinated Biphenyls: Dynamics of Dechlorinating Microorganisms and their Interactions with Methanogens and Sulfate Reducers

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The dynamics of PCB-dechlorinating microorganisms were determined along with sulfate reducers and methanogens using the most-probable-number technique. The time course Aroclor 1248 dechlorination mirrored the growth of dechlorinators. Dechlorination ensued as the dechlorinating population increased by two orders of magnitude from 2.45 × 10[3] to 4.59 × 10[5] cells/g sediment between 2 to 6 weeks. During this period PCB dechlorinating microorganisms dechlorinated Aroclor 1248 at a rate of 39.29 × 10[-3] mole Cl/g sediment/day and growth yield was 41.50 × 10[3] cells/mole Cl dechlorinated. Once dechlorination reached a plateau after 6 weeks, the number of dechlorinators began to decrease. On the other hand, dechlorinators inoculated into PCB-free sediments decreased in time from their initial level suggesting that PCBs are required for their selective enrichment. Sulfate reducers and methanogens increased in both PCB-free and contaminated sediments showing little difference between them. The potential role of methanogens and sulfate reducers on PCB dechlorination was investigated using specific inhibitors 2-bromoethanesulfonate (BES) and molybdate. Addition of molybdate had no effect on Aroclor 1248 dechlorination, indicating that sulfate reducers might not be directly involved in or inhibit the dechlorination process. In BES-amended sediments, *meta*-rich congeners such as 2,5,2',5'- 2,4,2',4'- and 2,5,2'-chlorobiphenyls were not dechlorinated. This suggests the selection of different dechlorinating populations. Interestingly, addition of both molybdate and BES completely inhibited Aroclor 1248 dechlorination.

Q-142. Selective Enrichment for PCB-Dechlorinating Anaerobes from Estuarine Sediments

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Bacterial enrichments developed from Baltimore Harbor sediments reductively dechlorinate polychlorinated biphenyls (PCBs) when incubated under anaerobic conditions. Initial enrichments produced various ortho, meta and para products from 2,3,4,5-chlorobiphenyl (CB) and 2,3,5,6-CB when maintained in estuarine or marine media. Successive transfer of these enrichments has resulted in selection of specific products. For example, initial enrichments with 2,3,5,6-CB expressed both meta and ortho dechlorination pathways but after sequential transfer on 2,3,5,6-CB only the ortho pathway remained. Initial enrichment with 2,3,4,5-CB resulted in para- and meta-dechlorination to 2,3,5-CB and 2,4,5-CB followed by ortho-, meta- and para-dechlorination to di- and monochlorobiphenyls. Successive transfer with 2,3,4,5-CB has lead to enrichments that only produce 2,3,5-CB, 3,5-CB and 2,5-CB with 3,5-CB being the main product. The specific activities observed in transfers on 2,3,5,6-CB and 2,3,4,5-CB were maintained regardless of the amount of sediment added to the medium. Continued transfer of all enrichment lines in the absence and presence of sediments is under examination. The effects of various carbon (energy) sources and inhibitors on dechlorination and enrichment/isolation will also be discussed.

Q-143.

Reductive Dechlorination of Coplanar PCB Congeners in the Anoxic Estuarine Sediment Slurries

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Of 209 PCB congeners, 20 congeners with chlorine atom at both *para* and *meta* positions but lack complete substitution in the *ortho* position show a coplanar configuration. It had been demonstrated that these coplanar congeners are more toxic and less biodegradable than nonplanar PCB congeners. Concern over their toxicity and bioaccumulation potential have emphasized the need to clean up these coplanar PCBs.

In this study, biodegradability of 4 coplanar congeners 3,3',4,4'-tetrachlorobiphenyl, 3,4,4',5-tetrachlorobiphenyl, 3,3',4,4',5-pentachlorobiphenyl, 3,3',4,4',5,5'-hexachlorobiphenyl were investigated by amending 10 ppm of each compound into the anoxic sediment slurries collected from the estuary of Tansui River and Er-Jen River. During 13 month incubation, the parent compounds and the intermediate products were determined with gas chromatography (GC) and GC-MS.

Except for 3,3',4,4',5,5'-hexachlorobiphenyl, all other tested coplanar congeners were dechlorinated in 10 month after a lag period of 61 days in the sediment slurries collected from Er-Jen River. However, both 3,3',4,4',5,5'-hexachlorobiphenyl and 3,3',4,4'-tetrachlorobiphenyl were persistent in the sediment slurries collected from Tansui River. Dechlorination of the other 2 congeners were much slower in the sediment slurries collected from Tansui River than those from Er-Jen River. Examination of the chromatograms over the time course of the incubation indicates that dechlorination of these congeners were initiated from *para* chlorine removal. One to three chlorines were removed from these congeners during 13 month incubation.

Q-144. Evidence of degradation and mineralization of biphenyl by anaerobic microbial consortium.

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In the past, degradation of biphenyl by aerobic microorganisms has been known, but information on its anaerobic degradation has been limited. We have previously developed an anaerobic microbial consortium in granular form that was shown to dechlorinate polychlorinated biphenyls (PCBs) into biphenyl. In this study, we demonstrate degradation and mineralization of biphenyl to CO_2 and CH_4 by these dechlorinating granules under methanogenic conditions. Biphenyl was degraded to p-cresol which was further mineralized to CO_2 and CH_4. These results were obtained with labeled ^{14}C-biphenyl as well as unlabeled biphenyl and p-cresol. Production of ^{14}C-CO_2 and ^{14}C-CH_4 was found to increase during a time course study. The ratio of ^{14}C-CO_2 and ^{14}C-CH_4 in the headspace was about 1:2 after 16 weeks of incubation. The tentative anaerobic biodegradative pathway of biphenyl is proposed as: biphenyl p-cresol CO_2 + CH_4. Our results indicate existence of novel biodegradative pathways in natural anaerobic microbial community that has broad implications in the field of microbial ecology and detoxification and elimination of toxic pollutants.

Q-145. Reductive dechlorination of an ortho-substituted PCB congener by Chesapeake bay sediments acclimated to para- and meta-chlorinated congeners

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On the spectrum of xenobiotic pollutants from easiest to most difficult, polychlorinated biphenyls (PCBs) are among the most challenging for bioremediation. Often dechlorination of meta- and para- chlorine moieties proceed at a faster rate than ortho-chlorine. Last year, we reported meta- and para-dechlorination of PCBs by sediments from the Chesapeake bay and recently, Berkaw et al., have reported reductive ortho-dechlorination of PCBs by estuarine sediments from the Baltimore Harbor. In order to further characterize our sediments, we examined the ability of our microbial consortia that had been acclimated to four concentrations of a meta- and para-substituted PCB congener to reductively dechlorinate an ortho-saturated PCB congener. Anaero-

Q-191 Low Temperature Microbial Aerobic Degradation of Polychlorinated Biphenyls in Sediment.
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Polychlorinated biphenyl (PCB)-contaminated upper Hudson River sediment amended with Aroclor 1242 was incubated at 4°C to confirm that microbial aerobic PCB degradation can occur at a low temperature. Congener-specific analysis of PCBs in the top 4 mm of the sediment showed loss of specific di- and trichlorobiphenyls within 70 days as compared to the same PCB congeners in the subsurface sediment. There was no loss of PCBs from the surface or subsurface of autoclaved sediment samples incubated at 4°C. The pattern of di- and trichlorobiphenyls lost from the surface of the sediment incubated at 4°C matched the pattern of microbial aerobic PCB degradation observed in sediment samples incubated at 25°C. These data indicate that low temperature microbial aerobic PCB degradation can occur in PCB-contaminated sediment.

Q-192 Biodegradation of Polychlorinated Biphenyls under Aerobic and Microaerophilic Conditions by Facultative Denitrifying, Microaerophilic Bacteria. J.H. LOBOS*. General Electric Corporate Research and Development, Schenectady, NY

Several novel PCB-degrading bacterial strains were isolated from Hudson River sediment contaminated with polychlorinated biphenyls (PCBs) after enrichment under microaerophilic conditions. Upon culturing in tubes containing a semi-solid agar medium, these strains demonstrated a preference for growth under microaerophilic conditions. In addition, these isolates are able to grow anaerobically under denitrifying conditions. Two of these strains were recently reported to be unable to grow on biphenyl as a sole carbon source, but appear to constitutively express the biphenyl dioxygenase genes even in the absence of PCBs or biphenyl (ASM Meeting, 1995). PCB biodegradation was examined under aerobic and microaerophilic conditions after these strains were grown aerobically or anaerobically under denitrifying conditions. The results indicate that these facultative microaerophilic, denitrifying strains are able to degrade PCBs under extremely low concentrations of oxygen (0.05 ppm O_2). Conventional methods for identifying these isolates has been inconclusive thus far. A 16S rRna analysis of these isolates is in progress. To my knowledge, this is the first report of aerobic or microaerophilic PCB-degrading bacterial strains capable of anaerobic growth under denitrifying conditions.

Q-193 Extraction and PCR-mediated amplification of microbial DNA from anaerobic PCB-dechlorinating enrichments that contain sediments or coal-based humic acids. M.A. ELBERSON[1]*, H.D. MAY[2], and K.R. SOWERS[1]. University of Maryland Biotechnology Institute, Baltimore, MD[1] and Medical University of South Carolina, Charleston, SC[2].

Para-, *meta-* and *ortho*-dechlorination activities of individual polychlorinated biphenyl congeners (PCBs) by estuarine sediments from Baltimore Harbor have been maintained throughout sequential transfers of enrichment cultures that contain up to 25% (wt/vol) petroleum based humic acids. However, microorganisms responsible for dechlorination have not yet been identified by standard isolation techniques. In order to identify PCB-dechlorinating microorganisms, a procedure was developed for the isolation and PCR amplification of DNA encoding 16S rRNA from marine and estuarine enrichments containing high concentrations of humic acids. Enrichment slurries were lysed with glass beads in phosphate buffer containing Denhardt's solution and the DNA was extracted with phenol-chloroform. Humic acids associated with DNA were removed with insoluble polyvinylpyrrolidone, extracted from a low-melt agarose gel containing soluble polyvinylpyrrolidone, then amplified by PCR using universal oligonucleotide primers. DNAs encoding 16S rRNA from Archaea (*Methanosarcina thermophila*), Bacteria (*Escherichia coli*), and Eucarya (*Saccharomyces cerevisiae*) were recovered and amplified from as few as 10^3 cells in humic acids slurry. This technique has been used to amplify and sequence genes encoding 16S rRNA from Baltimore Harbor enrichments that *para*-dechlorinate 2,3,4,5-PCB to 2,3,5-CB and *ortho*-dechlorinate 2,3,5-CB to 3,5-CB. The technique has also been used for analyses of *meta*-dechlorinating cultures from Hudson River that have been maintained in humic acids slurries. Initial analyses of the gene sequences obtained from these enrichments are presented.

Q-194

Cloning of *Corynebacterium sepedonicum KZA rcd* Gene Responsible for Reductive *ortho*-Dehalogenation of Halobenzoates and Construction of Coupled Reductive *Ortho*- and Hydrolytic *Para*-Dechlorination System for Degradation of PCBs

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Unlike oxygen-requiring pathways for degradation of (chloro)aromatic pollutants, reductive degradation/dechlorination has not been studied in molecular detail. Isolation of reductive dehalogenation genes provides an excellent model for studying anoxic degradation of halogenated aromatic xenobiotics. Reductive dechlorination of chlorobenzoate was implied in a few bacteria, but isolation of the corresponding genes has not been reported.

We have now cloned and expressed the *rcd* gene, encoding a novel CoA-, ATP-, Mg^{++}-, and NADH-dependent *ortho*-halobenzoate reductase/dehalogenase from the Gram positive bacterium *Corynebacterium sepedonicum* KZA. From a gene library of strain KZA, several independent recombinant plasmids were found that specify *ortho*-dehalogenation of 2-CBA and 2,4-DCB when complemented by the 4-CBA hydrolytic *para*-dechlorination *fcbABCD* operon we cloned previously from *A. globiformis* KZT1. The data indicate that reductive *ortho*-dechlorination requires both the *rcd* gene encoded dehalogenase/reductase and *fcbA* gene encoded chlorobenzoate CoA-ligase. The coding region for the *rcd* gene has been located within a 1.5 kb DNA fragment that is currently being sequenced.

Ortho- and *ortho+para*-chlorobenzoates are principal environmental products of anaerobic reductive dechlorination of PCBs followed by aerobic co-metabolism of the resulting low chlorinated biphenyls. The coupled reductive *ortho*- + hydrolytic *para*-dechlorination system we have engineered was introduced into biphenyl-degrader *C. testosteroni* 44, resulting in growth on *ortho* (+*para*)-chlorobiphenyl.

Q-195 Effects of Sub-Critical Micelle Concentrations of Surfactants on the Microbial Dechlorination of Polychlorinated Biphenyls.
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Surfactants have often been used in attempts to increase the biodegradation of sparingly soluble compounds like polychlorinated biphenyls (PCBs) but with mixed results. The goals of our present research are to determine if sub-critical micelle concentrations (CMCs) of surfactants enhance the microbial dechlorination of PCBs and if so, by what mechanism(s). We performed dechlorination assays with anaerobic sediment slurries spiked with 2,2',4,4',5,5'-hexachlorobiphenyl (245-245-CB) and inoculated with microorganisms eluted from Hudson River sediments, or spiked with Aroclor 1260 and inoculated with microorganisms eluted from Silver Lake sediments. Tween 80 and Triton X-705 were added at nominal concentrations equal to 5%, 25%, and 110% of their CMCs. Dechlorination of 245-245-CB was enhanced by Triton X-705, especially at 5% and 25% of its nominal CMC. At 25% of its CMC, Tween 80 slightly enhanced 245-245-CB dechlorination but had negligible effects at 5% or 110% of its nominal CMC. There were no detectable differences in the rate or extent of dechlorination of Aroclor 1260 among treatments. These results are discussed in terms of the partitioning behavior of the surfactants and their effects on PCB solubilization in sediment slurry systems.

Q-196 Dechlorination of PCBs in Soil inoculated with Anaerobic Bacterial Granules. M.R. NATARAJAN*, J. NYE, W. WU and M.K. JAIN. Michigan Biotechnology Institute, Lansing, MI.

The capability of anaerobic bacterial consortium developed in granular form to dechlorinate Aroclor 1254 present in soil was investigated. The contaminated soil (spiked with Aroclor 1254 at 500 mg/kg soil) incubated with the anaerobic granules under partially simulated anoxic conditions showed substantial dechlorination at room temperature. The congener specific analysis showed preferential dechlorination of higher chlorinated PCB congeners with production of lower-chlorinated compounds. No monochlorobiphenyl congeners were found to accumulate. Pretreated wood powder served as a suitable nutrient source to support the dechlorination. PCB dechlorination was very minimal in the absence of inoculation with the microbial granules. At 16 weeks of incubation, the homolog distribution of each PCB group confirmed further dechlorination of lower-chlorinated congeners produced at 8 weeks. This study demonstrates dechlorination of soil contaminated with fresh PCBs using exogenous anaerobic bacterial consortium. Results of this study show potential for use of these microbial granules by utility companies to bioremediate soils that becomes contaminated with PCBs by transformer blowouts.

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Q-186 Functions of Extracellular Polysaccharides of *Rhodococcus rhodochrous*. NORIYUKI IWABUCHI,† MICHIO SUNAIRI,† HISAO MORISAKI‡, and MUTSUYASU NAKAJIMA.†* †Nihon Univ., Fujisawa, Japan; ‡Ritsumeikan Univ., Kusatsu, Japan.

Rhodococcus is a versatile genus of nocardioform actinomycetes, which plays an important role for biodegradation of xenomaterials, *e.g.*, PCB. It is essential to understand its behavior in environments for the application to bioremediation. We report nature of the bacterial cell surface, *e.g.*, electrokinetic potential or hydrophobicity, which is an important determinant in the bacterial behavior.

Four colony-morphological mutants of *R. rhodochrous* (S-1, and S-2, mucoidal; R-1, and R-2, rough) produced 6.8, 14.5, 1.4, and 1.9 (mg dry EPS / g fresh cells), respectively. Their electrophoretic mobilities were almost the same negative values (-3×10^{-8} m^2/Vs) between pH 4 and 9.

Cell surface hydrophobicity was determined by five different methods, *i.e.*, MATH, contact angle, SAT, HIC and DOS. The order of hydrophobicity was determined as R-2 » R-1 » S-1 > S-2. LBM method devised for measuring cell surface hydrophobicity of mucoidal strains revealed that S-2 has hydrophobic surface covered with hydrophilic EPS, indicating that the EPS function as hydrophilin.

Next, the effect of hydrophilic EPS on adhesion of the bacterium to particles in environments was analyzed by model experiments using glass, quartz and teflon. Rough strains well adhered to the materials, whereas mucoidal strains little adhered to these materials. Sedimentation tests showed that the cells of rough strains settled within several hours, whereas mucoidal strains scarcely settled.

Mucoidal mutants appeared from rough strains, R-1 and R-2, at frequencies of 2.4×10^{-6} and 1.5×10^{-6}, respectively.

In conclusion, hydrophobic cells have the advantage for adhesion, in contrast, hydrophilic cells can be conveyed with movement of water.

Q-187 Integrating Surfactant Enhanced PCB Solubilization and Biodegradation in a Soil Remediation Process A. C. LAYTON,* J. P. EASTER, C. A. LAJOIE, M. MUCINNI, & G. S. SAYLER. University of Tennessee, Knoxville, TN 37932.

A two phase remediation process has been developed for polychlorinated biphenyl (PCB) contaminated soils at electric utility substations. In the first phase, 80-90% of the weathered Aroclor 1248 is desorbed from the soil in situ by a two day recirculating surfactant wash (1%wt/vol). In phase two, the surfactant/PCB solution is collected in a bioreactor and amended with nutrients and the field application vectors (FAVs) *Pseudomonas putida* IPL5::TnPCB and *Alcaligenes eutrophus* B30P4::TnPCB. These strains use the surfactant as a growth substrate and contain the entire PCB degradative operon inserted on a transposon. After 1 week, >90% of the surfactant and >30% of the PCBs are degraded. The residual desolubilized PCBs are deposited on a soild carrier and removed from the bioreactor effluent (>99%). The concentrated residual PCB congeners may be partially dechlorinated by physiochemical or biological processes and recycled to the bioreactor. Toxicity testing, using *Tetrahymena* and Microtox systems, is being performed on soils and process solutions. A proposed field trial will be performed at an electric power substation pending EPA approval.

Q-188 *In Situ* Biodegradation of PCB-Contaminated Surface Soils for Reduction of Leachable PCBs. M. J. R. SHANNON*, R. K. ROTHMEL, AND R. UNTERMAN. ENVIROGEN, INC. Lawrenceville, NJ 08648.

A two-year field demonstration of aerobic, *in situ* PCB bioremediation was completed. Two plots, each containing 3700 Kg of surface soil, were created within a greenhouse. The experimental (bioaugmentation) plot was dosed with biphenyl and PCB-degrading bacteria that exhibit complementary congener specificity (Type II and Type IV dioxygenase activities). The control plot received no cells and received a limited amount of biphenyl during the later part of the final year (biostimulation control).

The initial average PCB concentration of 39 mg/Kg was reduced by 44% to 22 mg/Kg in the experimental plot. During the 1994 season, 20% PCB degradation was achieved, most of which occurred during the first 4 weeks of treatment. Parallel laboratory experiments demonstrated that the limited PCB biodegradaqtion in 1994 was likely due to an insufficient amount of biphenyl. Additional biphenyl added in 1995 resulted in a further reduction in PCB concentration to 22 mg/kg.

Degradation during 1994 was limited to the lower chlorinated congeners (di- tri- and tetrachlorinated PCBs), and as the biological activity progressed during 1995 more extensive degradation of tetra- and pentachlorinated congeners occurred. Overall, 89% of the di-, 84% of the tri-, 51% of the tetra- and 28% of the pentachlorinated congeners were degraded by the end of the demonstration. The data show that biodegradation resulted in the destruction of soluble, bioavailable congeners, and suggest that biodegradation will result in PCB stabilization and reduced risk of PCB migration and exposure.

Q-189 Anaerobic *ortho* PCB Dechlorination by Estuarine and Marine Sediments. BERKAW[1], M., L. CUTTER[1], K. R. SOWERS[1], AND H. D. MAY[1]*. The Medical University of South Carolina, Charleston, SC[1], and the University of Maryland Biotechnology Institute, Baltimore, MD[1].

Estuarine sediments from Baltimore Harbor *ortho*-dechlorinate a number of PCB congeners under anaerobic conditions. *Ortho* dechlorination of 2,3,4,5-CB occurs with these sediments in marine, estuarine, and freshwater media. The effects of various media on the acclimation time and on the type of dechlorination that develops (*meta*, *ortho*, or *para*) are presented. Dechlorination (*meta*, *ortho*, and *para*) most rapidly develops (<1 month) in an estuarine medium lacking sulfate. Dechlorination is delayed in marine medium or by the addition of sulfate. Use of reduced anaerobic mineral medium (RAMM), a freshwater medium, delays the onset of *ortho* dechlorination for more than a month and heavily favors *para* dechlorination. *Ortho*-dechlorinating cultures have been maintained in the absence of sediment. After 3 serial transfers (the first containing supernatant from an active sediment) several transfer cultures *ortho* dechlorinated 2,3,5-CB after the *para* dechlorination of 2,3,4,5-CB. These cultures and their requirement for, or independence from, sediment are discussed. *Ortho* dechlorination has also been observed with sediments from other locations. Sediments from five sites in Charleston Harbor, one site in the Chesapeake Bay near the mouth of the Potomac River, and one site in the Hudson River (H7) were examined for *ortho* dechlorination in marine, estuarine, and freshwater media. *Ortho* dechlorination of 2,3,5-CB or 2,3,5,6-CB was observed with 3 of the 5 Charleston Harbor sediments, however none of these developed activity as quickly as Baltimore Harbor sediments do and the type of dechlorination varies with the site and environmental conditions. The dechlorination activities expressed by sediments from all these sites are presented.

Q-190
Identification of Plants Having Potential Rhizosphere Effects on Polychlorinated Biphenyl Biodegradation. ERIC S. GILBERT* and DAVID E. CROWLEY. Univ. of Calif., Riverside, CA 92521.

The rhizosphere microenvironment has been reported to enhance the biodegradation of xenobiotic chemicals. The potential for a rhizosphere effect on polychlorinated biphenyl (PCB) biodegradation has not been fully evaluated. As part of a study of rhizosphere influence on PCB biodegradation, a screening assay was developed to identify plants which might induce bacterially-mediated PCB degradation.

Arthrobacter sp. strain B1B, a Gram-positive bacterium known to cometabolize Aroclor 1254, was grown on selected plant extracts. Washed cell suspensions of strain B1B were prepared; 4,4'-dichlorobiphenyl subsequently was added and the rate of formation of the phenylhexadienoate ring-fission product, an indicator of PCB oxidation, was monitored spectrophotometrically. Rates of product formation after growth on plant substrates were compared to rates after growth on biphenyl, the non-chlorinated PCB analog, and on various nutrient media.

Root extracts of common plants such as rye grass (*Lolium perenne*) and green bean (*Phaseolus vulgaris*) did not stimulate ring-fission product formation, nor did compost extracts. However, a representative aromatic plant, *Mentha sp.*, proved to be an effective inducer of ring-fission product formation. 4-chlorobenzoate was identified by HPLC as a metabolite, indicating hydrolysis of the ring-fission product also occurred. These results suggest that certain plants may produce metabolites which, if present in the rhizosphere, may promote PCB cometabolism.